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Molecular characterization and evolution dynamics of influenza B viruses circulating in Germany from season 1996/1997 to 2019/2020

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ABSTRACT

Influenza B viruses are responsible for significant disease burden caused by viruses of both the Yamagata- and Victoria-lineage. Since the circulating patterns of influenza B viruses in different countries vary we investigated molecular properties and evolution dynamics of influenza B viruses circulating in Germany between 1996 and 2020. A change of the dominant lineage occurred in Germany in seven seasons in over past 25 years. A total of 676 sequences of hemagglutinin coding domain 1 (HA1) and 516 sequences of neuraminidase (NA) genes of Yamagata- and Victoria-lineage viruses were analyzed using time-scaled phylogenetic tree. Phylogenetic analysis demonstrated that Yamagata-lineage viruses are more diverse than the Victoria-lineage viruses and could be divided into nine genetic groups whereas Victoria-lineage viruses presented six genetic groups. Comparative phylogenetic analyses of both the HA and NA segments together revealed a number of inter-lineage as well as inter- and intra-clade reassortants. We identified key amino acid substitutions in major HA epitopes such as in four antigenic sites and receptor-binding sites (RBS) and in the regions close to them, with most substitutions in the 120-loop of both lineage viruses. Altogether, seventeen substitutions were fixed over time within the Yamagata-lineage with twelve of them in the antigenic sites. Thirteen substitutions were identified within the Victoria-lineage, with eleven of them in the antigenic sites. Moreover, all Victoria-lineage viruses of the 2017/2018 season were characterized by a deletion of two amino acids at the position 162–163 in the antigenic site of HA1. The viruses with triple deletion Δ162–164 were found in Germany since season 2018/2019. We highlighted the interplay between substitutions in the glycosylation sites and RBS and antigenic epitope during HA evolution. The results obtained underscore the need for continuous monitoring of circulating influenza B viruses. Early detection of strains with genetic and antigenic variation is essential to predict the circulation patterns for the following season. Such information is important for the development of optimal vaccines and strategies for prevention and control of influenza.

1. Introduction

Influenza is one of the most important infectious diseases and contributes to increased morbidity and mortality globally. Traditionally, most influenza reports focus on influenza A, despite influenza B carries large impact in public health and is associated in particular with severe disease in children (McCullers and Hayden 2012; Sakudo et al., 2012) but also in adults as reflected by the unusually severe influenza B season of 2017/2018 which caused the highest excess-mortality of the last two decades in Germany (Robert-Koch-Institut 2018).

The first influenza B virus was isolated in 1940 and since the late 1980s, influenza B viruses diverged and have been divided into the B/Victoria/2/87- and B/Yamagata/16/88-lineage characterized by different genetic and antigenic features (Rota et al., 1990; Shaw et al. 2002). Victoria-lineage viruses were predominant and circulated worldwide in the late 1980s whereas the Yamagata-lineage became dominant in the early 1990s (Rota et al., 1992). Victoria-lineage viruses reappeared in Europe and North America during the 2001/2002 season (Shaw et al. 2002). It has been found that the majority of these viruses were reassortants having a Victoria-like hemagglutinin (HA) and a Yamagata-like neuraminidase (NA) (Shaw et al. 2002). Since 2001/2002, both lineages of influenza B viruses co-circulate in Germany and worldwide. However, the proportion of influenza B viruses from each lineage circulating can vary by geographic region (Chen and Holmes 2008; ECDC/WHO 2018; FluNet; Garten et al., 2018; Kuo et al., 2016; Puzelli et al. 2004; Tewawong et al., 2017).

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Both HA and NA genes encoding the surface glycoproteins are important for influenza virus infectivity and immune response. The HA1 genomic region is known to contain the HA head domain which is inclined to frequent mutations to evade the binding of protective antibodies against influenza viruses (Shen et al., 2009; Wang et al., 2008). Amino acid sequence variations in the HA surface glycoprotein affected both antigenic and receptor-binding sites (RBS). Four major antigenic epitopes are located close in space and form a large continuous antigenic site: the 120-loop (positions 116–137), the 150-loop (141–150), the 160-loop (162–167) and the 190-helix (194–202) and their surrounding regions (Shen et al., 2009; Wang et al., 2008). RBS is located at the top of the HA1 membrane-distal domain and formed by 190-helix (positions 193 to 202) at the top, the 240-loop (237 to 242) as the left edge, and the 140-loop (136 to 143) as the right edge (Wang et al., 2007). It was also reported that transitions between influenza antigenic clusters are often associated with substitutions at sites near the RBS (Wang et al., 2008) . HA1 regions 73–79, 139–152 and 226–241 are part of the antigenic site of influenza B virus HA surrounding the RBS. Structural plasticity of these regions is assumed to be an important factor for efficient binding of antibodies and receptors (Wang et al., 2008).

N-linked glycosylation plays an important role in stabilizing the HA structure, to protect the HA protein from enzymatic hydrolysation and to evade antibody recognition (Wei et al. 2010). The diversity in glycosylation on the HA1 epitope is known to result in an antigenic change (Wang et al., 2008).

Additionally, a number of the amino acids with high Shannon entropy not only in the antigenic site, but also outside it could contribute to the antigenic properties (Shannon 1948; Suptawiwat et al., 2017). In information theory, Shannon entropy is a parameter which determines how unpredictable is a message. If a position is totally conserved and can be predicted with 100% certainty, the entropy at that position is zero (Shannon 1948). Shannon entropy has been used to map antigenic residues of influenza A HA (Deem and Pan 2009). Suptawiwat et al. (2017) used this approach for influenza B .

NA has multiple functions in the life cycle of influenza virus, especially in the late stage of virus replication. Amino acid changes prevalent on the top of the NA indicate that this is the position of antigenic sites indicating that immune selection plays an important role in the NA evolution (Air et al., 1990; Burmeister et al., 1992; Colman et al., 1983).

Influenza B virus undergoes antigenic drift through cumulative mutations and antigenic variation and through genetic reassortment among co-circulating strains of different lineages (McCullers et al., 1999; Puzelli et al. 2004; Tewawong et al., 2017). Influenza B viruses are not divided into subtypes, but can be further classified into specific clades and genetic groups within both lineages. However, it should be noted that the amino acid substitutions associated with the division of clades or genetic groups do not always have a notable effect on antigenicity (ECDC 2011; ECDC 2013; ECDC 2018).

Reassortment plays an important role in the evolution of influenza B virus producing a new reassortant genome with optimized match of the HA and NA functional properties (Chen and Holmes 2008). This allows influenza B virus to escape host immunity and continue to evolve without formation of subtypes (McCullers et al., 1999). Moreover, some insertions and deletions increase the genetic diversity of influenza B viruses and may also contribute to alterations in the antigenic characteristics (Chen and Holmes 2008; McCullers et al., 1999). Few investigations reported on large-scale evolutionary dynamics of influenza B viruses at genome-wide level (Bedford et al. 2015; Chen and Holmes 2008; Langat et al. 2017; Vijaykrishna et al. 2015). Langat et al. (2017) showed that global phylodynamics and epidemiologic interactions of influenza B viruses were shaped by reassortment, genomic compatibility and differing patterns of antigenic change. Notably, the circulating patterns of influenza B viruses may vary in different countries (Kuo et al., 2016; Oong et al. 2017; Sam et al. 2015; Tan et al. 2013; Tewawong et al., 2015).

Continuous antigenic variation and antigenic drift of influenza B viruses request close monitoring of circulating viruses for an optimal vaccine composition. In a mismatched season, influenza vaccine effectiveness may be suboptimal, potentially leading to an increased public health burden during those seasons (Heikkinen et al., 2014; Ray et al., 2017; Skowronski et al. 2014). In Germany, the trivalent inactivated vaccine has been used till 2018/2019 as recommended by World Health Organization (WHO) for seasonal influenza. Since 2018, the vaccination has been carried out using an inactivated, quadrivalent vaccine (STIKO 2018). Older adults and people with chronic diseases have an increased risk of developing severe influenza and are target groups for seasonal influenza vaccination. The WHO and National Immunization Technical Advisory Groups in most industrialized countries including The Standing Committee on Vaccination (STIKO) in Germany recommend seasonal influenza vaccination for these groups (Michaelis et al. 2021; STIKO 2018). Vaccination coverage in Germany among the people aged $\geq$ 60 years averages between 36% and 51% depending on the season (Rieck et al., 2021). In the season 2020/21 e.g., a nationwide vaccination rate of 47.3% was achieved. The vaccination rate in Germany differs in the eastern and western part of the country and is in the eastern Federal states (OBL) far above the vaccination rate of western federal states (WBL). In 2020/21, the vaccination rate among persons aged at least 60 in the WBL was 44.0% (range 30.4–58.0%) compared to the OBL with 62.3% (range 58.9–67.5%) (Rieck et al., 2021). Data on vaccination coverage of children in Germany are not available as they are not a target group for seasonal influenza vaccination. However, STIKO recommends the flu vaccination for children at increased risk of complications because they suffer from certain previous illnesses. For children from 2 to 17 years of age a live vaccine is also available (STIKO 2021).

We traced the evolutionary dynamics of influenza B viruses circulating in Germany during the past 25 years. For this we used time-scaled phylogenetic analysis and estimated the rates of nucleotide substitution for HA and NA of both type B lineages. The HA (HA1 genomic region) and NA gene pattern of changes were studied and substitutions along the "trunk" branches of the phylogenies identified. Also, we analyzed interconnection between substitutions in the glycosylation and receptor-binding sites and antigenic sites during HA evolution.

2. Materials and methods

2.1. Ethics statement

Clinical samples were taken from patients with influenza-like illness as part of the national influenza surveillance in Germany and sent to the National Reference center for Influenza at the Robert Koch Institute (Berlin, Germany). The medical practices were located all over Germany. The analyses of all data were done anonymously. Influenza sentinel surveillance is covered by German legislation (§13, §14, Protection against Infection Act).

2.2. Clinical samples

Viral specimens (nasal and/or throat swabs) were collected since season 1996/1997. Swabs were transported in 1 ml UTM-RT medium (Copan, Murrieta, USA) and upon arrival adjusted to 3 ml with sterile minimal essential medium with HEPES (GibcoBRL, Eggenstein, Germany) and 100 U/ml penicillin–streptomycin (Gibco) and aliquoted.

2.3. RNA extraction, cDNA synthesis and sequencing

Viral RNA was extracted from 200 µl diluted clinical specimen using the MagNA Pure 96 DNA and Viral NA Small Volume Kit (Roche Deutschland Holding GmbH, Mannheim, Germany). cDNA synthesis was performed using 25 µl RNA and 15 µl reaction mixture containing dithiothreitol (DTT, 2.5 mM, Invitrogen, Karlsruhe, Germany), deoxynucleotide triphosphates (dNTPs, each of 200 µM, Invitrogen), random hexamer primers (500 nM, Metabion, Martinsried, Germany), 1 U

RNAsin (Promega, Madison, WI) and 5 U moloney murine leukemia virus (M-MLV) reverse transcriptase in first-strand buffer (Invitrogen). RT reaction was performed under the following conditions: 42 °C (5 min), 37 °C (30 min) and 95 °C (5 min).

PCR was performed using 2.5 µl cDNA and a reaction mixture including $MgCl_2$ (2 mM, Invitrogen), dNTPs (each of 200 µM, Invitrogen), 0.5 U Platinum®Taq DNA polymerase in PCR buffer (Invitrogen) and primers (each of 750 nM, primer sequence on request). Finally, the PCR mixture was adjusted to a total volume of 25 µl with H_2O. The general PCR conditions included 45 cycles of 94 °C for 30 s, 54–60 °C for 30 s, and 72 °C for 3 min. Each PCR fragment was gel extracted and purified before being sequenced using the respective PCR primers.

PCR products of amplified HA and NA segments were sequenced by automated nucleotide cycle sequencing (primer sequence on request) using the BigDye®Terminator v3.1Cycle Sequencing Kit (Applied Biosystems, Darmstadt, Germany) and a capillary sequencer 3130xl (Applied Biosystems).

Influenza B HA and NA gene nucleotide sequences were deposited in GISAID (Tables S1 and S2).

2.4. Sequence data

The sequences were analyzed using BioEdit (version 7.2.5.0), DNASTAR SeqMan Pro (version 10.0.1) or Geneious 2020.2. Sequences generated in this study are available in GISAID repository (http://gisaid.org) under accession numbers listed in Tables S1 and S2.

2.5. Datasets

To produce time-scaled phylogenetic tree we used 245 (from 341) sequences of HA1coding domain and 191 (from 258) sequences of NA genes of Yamagata-lineage viruses. Similarly, we selected 248 (from 335) sequences of HA1 coding domain and 209 (from 258) sequences of NA genes of Victoria-lineage viruses. These HA and NA sequences were selected to analyze all seasons in which influenza B viruses circulated. We excluded viruses circulating in the same season that had very similar sequences. For this analysis we used sequences obtained from clinical specimens and excluded sequences of viruses isolated in cell culture.

Large-scale investigations have shown that recombination is absent or exceedingly rare in influenza viruses (Boni et al., 2008). Nevertheless, we checked all alignments for evidence of recombination using RDP4 (Martin et al., 2015). No recombinant sequence was identified.

2.6. Phylogenetic analyses

We first ran maximum-likelihood analyses on all datasets using PhyML v3 with smart model selection (PhyML-SMS) using the Bayesian information criterion and a tree search using subtree pruning and regrafting (Guindon et al., 2010, 2005; Lefort et al., 2017). Branch robustness was estimated using Shimodaira-Hasegawa-like approximate likelihood ratio tests (SH-like aLRT) (Anisimova et al., 2011). We then confirmed the presence of strong temporal signal in all datasets by producing a regression of root-to-tip distances versus time using TempEst v1.5.1 (Rambaut et al., 2016).

In order to generate time trees and estimates of evolutionary rates, we ran Bayesian Markov chain Monte Carlo (BMCMC) runs using BEAST v1.10.4 (Suchard et al., 2018). For each alignment, we used the nucleotide substitution model identified by PhyML-SMS, a strict molecular clock model and a coalescent demographic model assuming constant population size. The output of multiple BMCMC runs was examined for convergence and appropriate sampling of the posterior using Tracer v1.7.1 (Rambaut et al., 2018), before being merged using LogCombiner v1.10.4 (distributed with BEAST). The maximum clade credibility (MCC) tree was identified from the posterior set of trees (PST) and annotated with TreeAnnotator v1.10.4 (distributed with BEAST). Branch robustness was estimated based on their posterior probability in the PST. We used iTol v4 to work on the display of MCC trees (Letunic and Bork 2019) and used hue colors found from top to bottom: https://www.w3schools.com/colors/colors_picker.asp

3. Results

3.1. Co-circulation of the Yamagata- and Victoria-lineage viruses

In Germany, both lineages of influenza B viruses co-circulated with varying prevalence in most seasons (Table 1).

In Germany, circulating influenza B viruses had lineage mismatch with the trivalent vaccine recommended by WHO for six times. The seasons 2002/2003, 2005/2006, 2008/2009, 2012/2013, 2015/2016, 2016/2017 and 2019/2020 were characterized by a change of the dominant influenza B lineage compared to the previous season. Such a drastic change of lineage occurred seven times during the last 25 years.

3.2. Time–scaled phylogenetic tree for HA1 and NA genes of influenza B viruses

To evaluate features of the evolution dynamics of influenza B viruses circulating recently in Germany we generated the time-scaled phylogenetic tree of the HA1 and NA genes of Yamagata- and Victoria-lineage viruses identified between 1996/1997 and 2019/2020 (Figs. 1–4). HA and NA genes of both lineages could be divided into genetic groups differing by amino acid substitutions. We focused on amino acid substitutions on the 'trunk' of the phylogenetic tree and on substitutions determining genetic groups because these changes were fixed in the virus population (Figs. 5–8).

It should be noted that direct Sanger sequencing of the HA1 domain and NA was carried out from randomly selected seasonal samples which were tested positive for influenza B virus immediately after sampling, starting from the 1996/1997 influenza season. Here, phylogenetic analyses are based on consensus nucleotide sequences because deep sequencing as Next Generation Sequencing (NGS) was not available.

3.2.1. Yamagata-lineage

HA of Yamagata-lineage viruses could be divided into nine genetic groups: Yamanashi1998, Sichuan1999, HongKong2000, Egypt2005, Florida2006, Brisbane2007, Massachusetts2012, Bangladesh2007 and Phuket2013 (Figs. 1 and 5 and Table 2). The Yamagata-like viruses showed a complex tree topology. Most viruses evolved in different genetic groups at the same time. A higher degree of homogeneity was only observed in a few seasons (Table 2). Thus, viruses of 1996/1997 and 2010/2011 seasons belonged exclusively to the genetic group Yamanashi1998 and Bangladesh2007, respectively, and all viruses circulating since 2013/2014 belonged to the genetic group Phuket2013 that belongs to the clade 3 (Y3) in accordance with WHO classification introduced in 2011 and 2021 (ECDC 2011, 2021). According to this classification, the genetic groups Brisbane2007 and Massachusetts2012 belong to clade 2 (Y2) and genetic group Bangladesh2007 – to clade 3 (Y3).

More diversity was seen in all other seasons analyzed (Table 2). Yamagata-lineage viruses of the season 1998/1999 belonged either to the genetic group Yamanashi1998 or genetic group Sichuan1999. Viruses circulating in 2001/2002 belonged either to the Sichuan1999 or HongKong2000 genetic group, and the viruses of the season 2004/2005 belonged either to the Egypt2005 or Brisbane2007 group. Viruses circulating during 2005/2006 were found in two genetic groups: Brisbane2007 and Bangladesh2007 as well as the viruses of 2006/2007 that were found in Florida2006 and Brisbane2007 groups. Viruses of 2007/2008 season represented even three genetic groups: Florida2006, Brisbane2007 and Bangladesh2007. Similarly, the 2011/2012 viruses were detected also in three genetic groups: Massachusetts2012, Bangladesh2007 and Phuket2013. Finally, the viruses from 2012/2013

Table 1
Distribution between Yamagata- and Victoria-lineages of influenza B viruses circulated in Germany from season 1996/1997 to 2019/2020 and number of HA and NA gene sequences analyzed.

Influenza season	Influenza B (%) *	Share of Yamagata (%)	Share of Victoria (%)	Recommended virus for trivalent vaccine (lineage)	Yamagata number of sequences HA/NA	Victoria number of sequences HA/NA
1996/1997	54	100	0	B/Beijing/184/1993 (Yam**)	12/6	
1997/1998	0	0	0	B/Beijing/184/1993 (Yam)		
1998/1999	33	100	0	B/Beijing/184/1993 (Yam)	9/13	
1999/2000	<1			B/Beijing/184/1993 (Yam)	1/0	
2000/2001	4	100	0	B/Beijing/184/1993 (Yam)	8/14	
2001/2002	53	80	20	B/Sichuan/379/1999 (Yam)	30/23	9/9
2002/2003	14	**0**	**100**	B/Hong Kong/330/2001 (Vic***)		23/6
2003/2004	1			B/Hong Kong/330/2001 (Vic)		
2004/2005	20	25	75	*B/Shanghai/361/2002 (Yam)*	4/4	6/6
2005/2006	70	**95**	**5**	B/Shanghai/361/2002 (Yam)	3/3	15/25
2006/2007	2	50	50	B/Malaysia/2506/2004 (Vic)	2/2	1/1
2007/2008	48	99	1	*B/Malaysia/2506/2004 (Vic)*	27/7	2/0
2008/2009	21	**2**	**98**	*B/Florida/4/2006 (Yam)*	1/0	9/3
2009/2010	<1			B/Brisbane/3/2007 (Vic)		3/0
2010/2011	37	14	86	B/Brisbane/3/2007 (Vic)	10/10	36/33
2011/2012	24	18	82	B/Brisbane/3/2007 (Vic)	14/13	33/33
2012/2013	35	**90**	**10**	B/Wisconsin/01/2010 (Yam)	12/13	3/6
2013/2014	9	80	20	B/Massachusetts/02/2012 (Yam)	2/4	2/4
2014/2015	31	98	2	B/Massachusetts/02/2012 (Yam)	45/30	2/2
2015/2016	55	**3,5**	**96,5**	*B/Phuket/3073/2013 (Yam)*	13/3	146/86
2016/2017	6	**80**	**20**	*B/Brisbane /60/2008 (Vic)*	45/12	10/7
2017/2018	68	99	1	*B/Brisbane /60/2008 (Vic)*	102/100	6/6
2018/2019	<1			B/Colorado/6/2017 (Vic)		1/1
2019/2020	15	**5**	**95**	B/Colorado/6/2017 (Vic)	1/1	28/30
Total:					341/258	335/258

Influenza B (%) *
percentage against influenza A.
Yam** Yamagata-lineage.
Vic*** Victoria-lineage.
Mismatch of the dominant lineage with the trivalent vaccines recommended by the WHO is labeled in italics. Change of the dominant lineage compared to the previous season is labelled in a bold font.

were identified in the genetic groups Massachusetts2012 and Bangladesh2007.

Distribution of Yamagata-lineage viruses NA is not fully congruent with the HA phylogeny of the same viruses (Figs. 2, 6 and Table 2). NA of Yamagata-lineage viruses could be divided into eight genetic groups, because the HA-genetic group Florida2006 is in the NA phylogenetic tree a part (genetic subgroup) of the Brisbane2007 genetic group (Table 2). Further, HA of Wisconsin-like viruses belongs to the genetic group Phuket2013, while NA of the same viruses belongs to the genetic group Bangladesh2007. For this reason, the viruses circulating during 2011/2012 were distributed not in three (as HA) but in two genetic groups: Massachusetts2012 and Bangladesh2007 (Table 2).

3.2.2. *Victoria-lineage*

The HA of these viruses could be divided into six genetic groups: HongKong2001, Brisbane2002, Malaysia2004, HongKong2009, Brisbane2008, Norway2015. The genetic subgroup Ohio2005 is the part of the genetic group Brisbane2002, and the genetic subgroups Colorado2017 and Washington2019 are part of the genetic group Norway2015 (Figs. 3, 7 and Table 2). In contrast to the HA of the Yamagata-lineage, the phylogeny of the HA of the Victoria-lineage viruses was characterized by a single tree 'stem' and most viruses evolved only in one genetic group. Thus, all viruses circulating during 2002/2003 belonged only to the genetic group Brisbane2002. The viruses circulating in 2004/2005 belonged exclusively to the genetic subgroup Ohio2005 (Table 2). All viruses of 2005/2006, 2006/2007 and 2007/2008 seasons were found in the Malaysia2004 group.

Viruses of the season 2008/2009 represented exclusively the genetic group Brisbane2008 belonging to clade V1A while viruses of the next seasons 2009/2010 and 2010/2011 belonged to the two genetic groups, HongKong2009 (clade V1B) and Brisbane2008 (clade V1A), according to the WHO clade classification (ECDC 2013, 2021). Notably, one virus isolated in 2011 was found in the genetic group Malaysia2004. Further, all viruses identified in 2012/2013 and 2013/2014 represented exclusively the group Brisbane2008.

The viruses circulating during 2014/2015, 2015/2016 and 2016/2017 formed the genetic group Norway2015 and the viruses of the season 2017/2018 – the genetic subgroup Colorado2017 (V.1A.1) within the genetic group Norway2015. Finally, the viruses of 2018/2019 and 2019/2020 seasons belonged to the genetic subgroup Washington 2019 (V1A.3) (ECDC 2020, 2021), also within the genetic group Norway2015.

The NA gene phylogeny of Victoria-lineage viruses was not fully congruent with the HA phylogeny (Figs. 4, 8 and Table 2). NA of Victoria-lineage viruses could be divided into seven genetic groups, because the HA-genetic subgroup Ohio2005 became a separate genetic group within the NA phylogenetic tree. Thus, the NA of the viruses circulating during 2004/2005 clustered within the Malaysia2004 group, while the HA of these viruses belonged to the subgroup Ohio2005. Further, the NA of viruses from 2002/2003 belonged to the genetic group Brisbane2002 and Malaysia2004, while the HA of these viruses represented exclusively the genetic group Brisbane2002. Finally, the NA of all viruses from 2005/2006 and NA of one virus isolated in 2006/2007 as well as one virus isolated in 2012 represented the Ohio2005 group while the HA of these viruses (with exception of the virus isolated in 2012) belonged to the group Malaysia2004 (Table 2).

3.3. *Substitutions occurring in the neutralizing epitopes and glycosylation sites of hemagglutinin*

Each genetic group was characterized by emergence of amino acid substitutions either in the antigenic site (120-loop, 150-loop, 160-loop) or in the 190-helix that is an antigenic site and RBS simultaneously, or in positions surrounding the RBS or in positions with high Shannon entropy values described by Suptawiwat et al. (2017). Substitutions described here were generally compared for the Yamagata-lineage to the

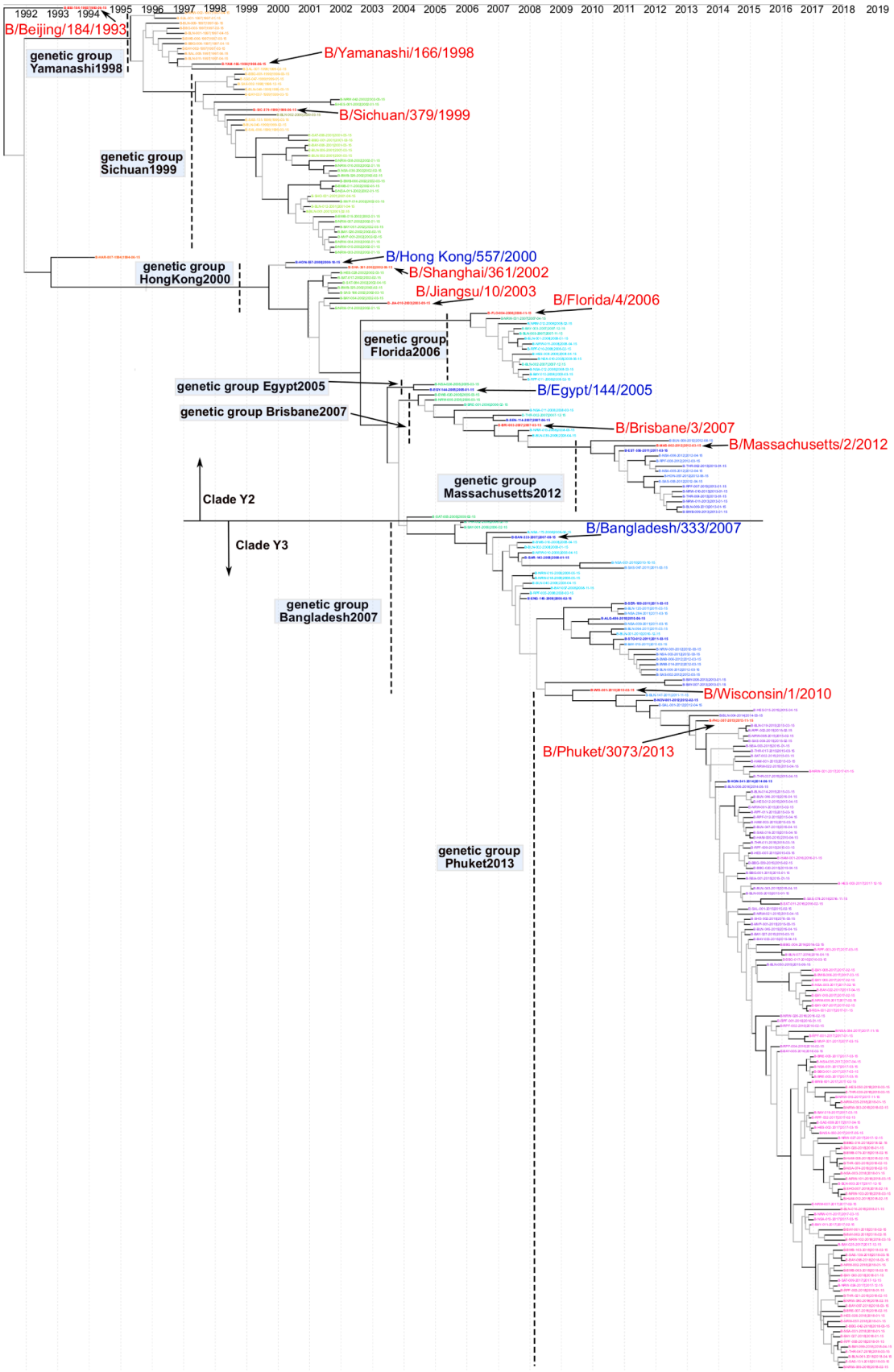

Fig. 1. Maximum clade credibility tree inferred from 245 Yamagata-lineage HA1 gene sequences. Branches of the phylogeny are colored according to the season and time of isolation of viruses. Vaccine viruses are colored in red, reference viruses are colored in blue. Genetic groups are indicated by a dotted line.

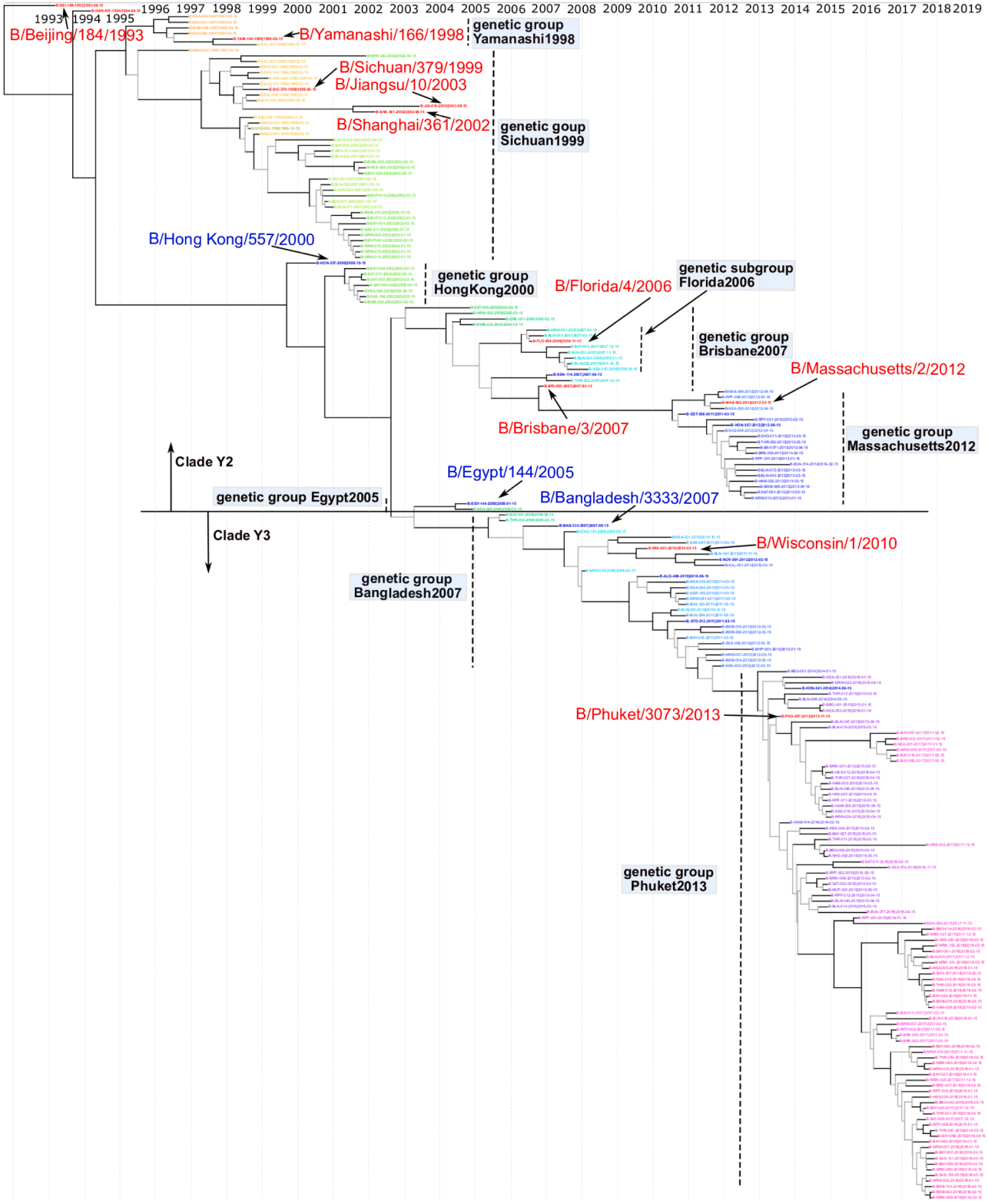

Fig. 2. Maximum clade credibility tree inferred from 191 Yamagata-lineage NA gene sequences. Branches of the phylogeny are colored according to the season and time of isolation of viruses. Vaccine viruses are colored in red, reference viruses are colored in blue. Genetic groups are indicated by a dotted line.

vaccine virus B/Beijing/184/1993 and for the Victoria-lineage to the vaccine virus B/Hong Kong/330/2001 (Figs. 9 and 10).

3.3.1. *Yamagata-lineage*

Altogether, seventeen substitutions were fixed over time, twelve of them in the antigenic sites or close to it: two in the 120-loop (N116K, N126D) and three substitutions in its vicinity (K48R, N56D, K182E), two in the 150-loop (R149K, S150I), three in the 160-loop (R162K, N165Y, T167N), and two in the 190-helix (I198T and N202S) (Fig. 9). Replacement at antigenic sites was also accompanied in some seasons by emergence of potential glycosylation sites, e.g. I198T in 1997 and T167N in 2002 (Fig. 9).

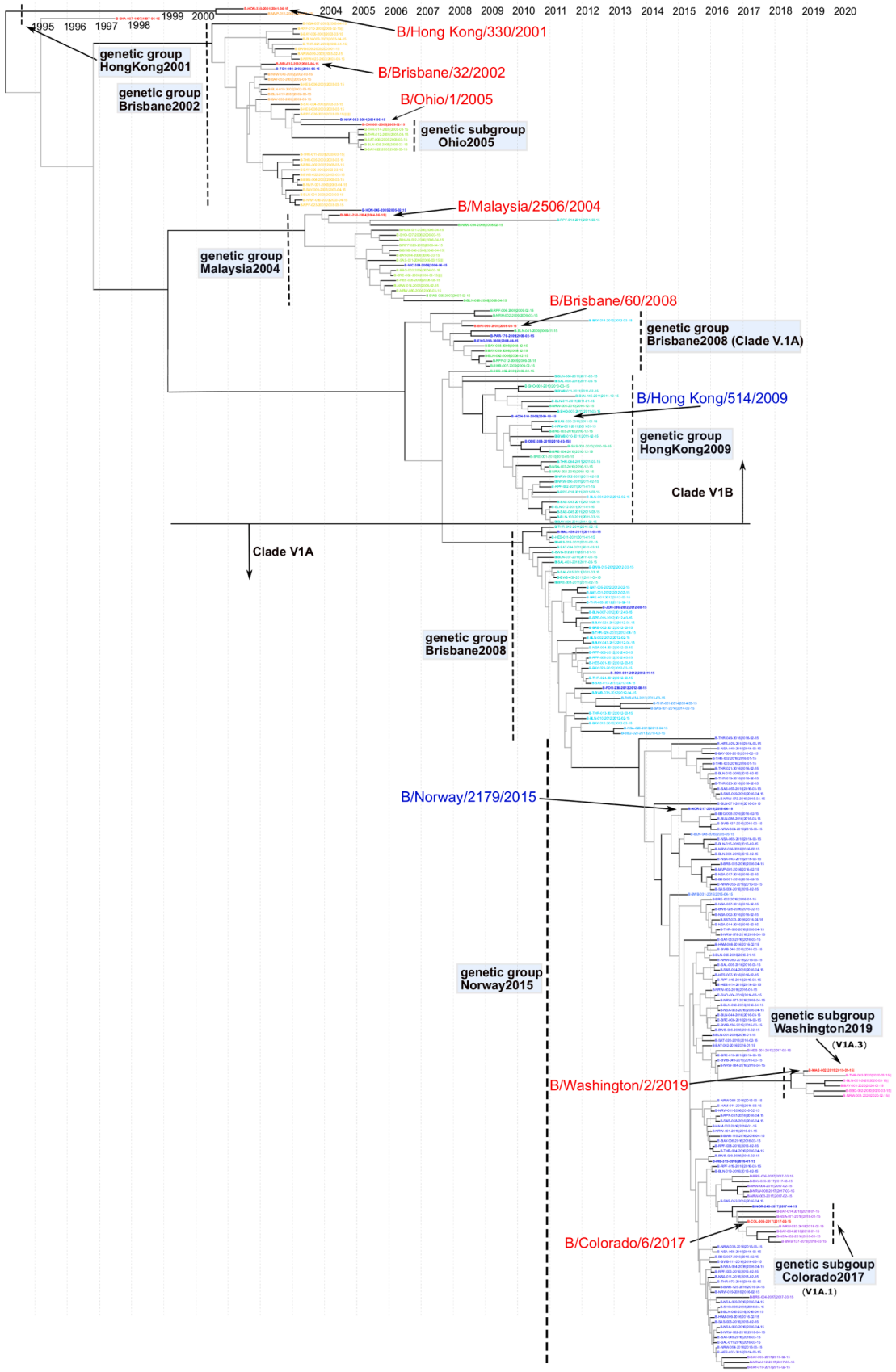

Fig. 3. Maximum clade credibility tree inferred from 248 Victoria-lineage HA1 gene sequences. Branches of the phylogeny are colored according to the season and time of isolation of viruses. Vaccine viruses are colored in red, reference viruses are colored in blue. Genetic groups are indicated by a dotted line.

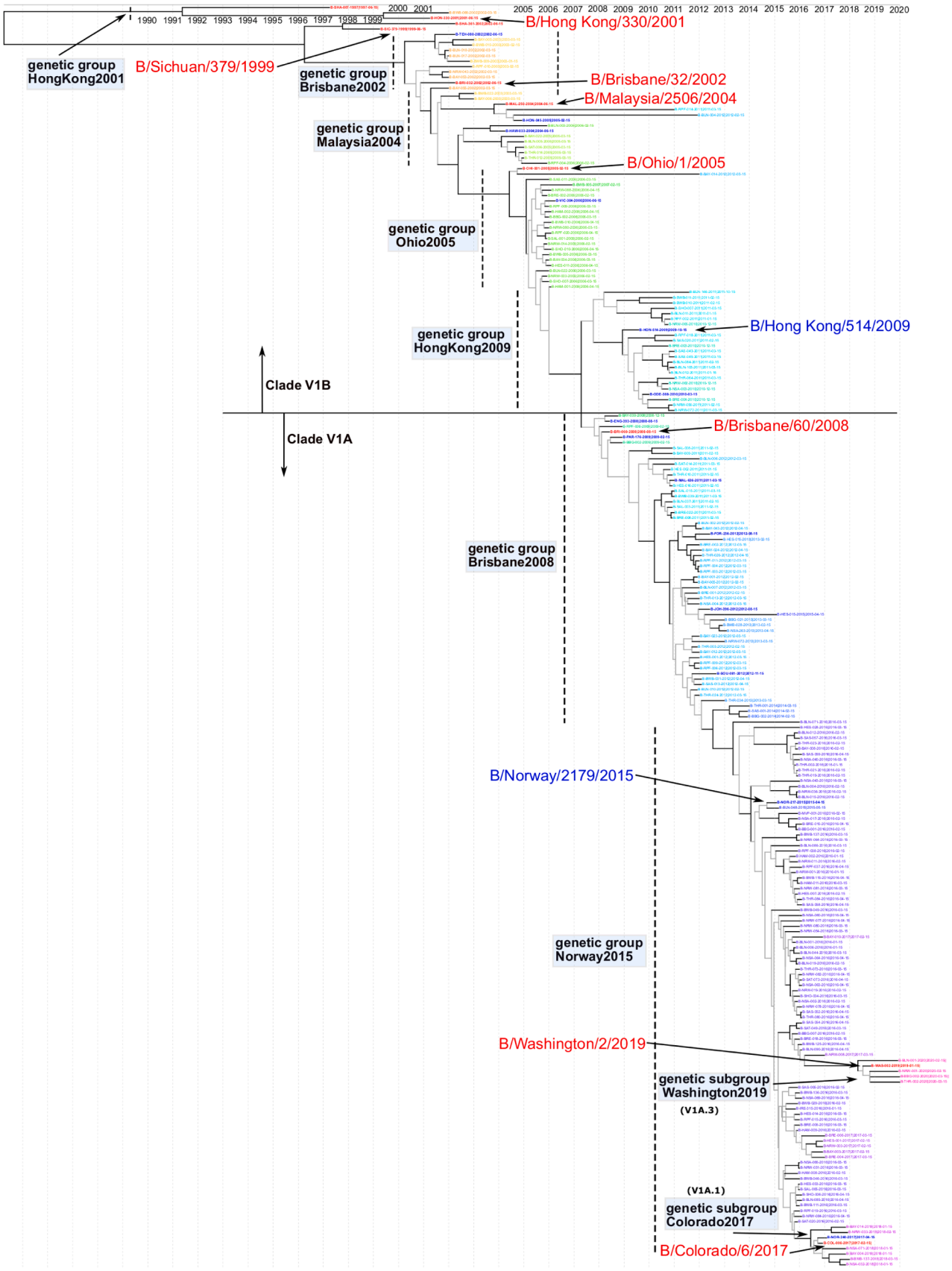

Fig. 4. Maximum clade credibility tree inferred from 209 Victoria-lineage NA gene sequences. Branches of the phylogeny are colored according to the season and time of isolation of viruses. Vaccine viruses are colored in red, reference viruses are colored in blue. Genetic groups are indicated by a dotted line.

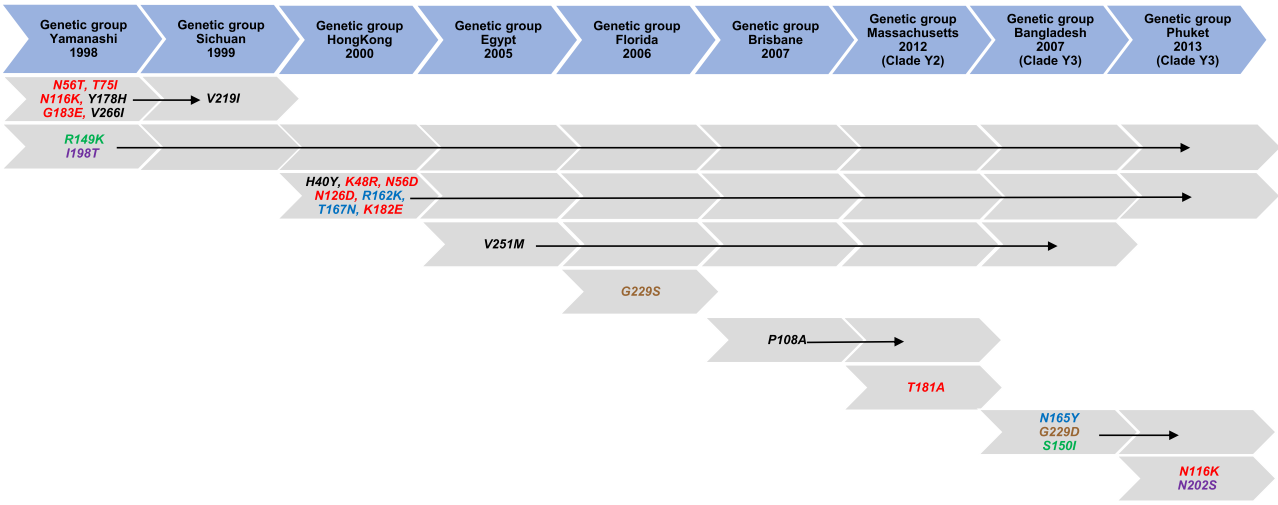

Fig. 5. Amino acid substitutions occurring along the phylogenetic 'trunk' and determining genetic group distinctions of HA1 of Yamagata-lineage viruses (compared with B/Beijing/184/1993). Amino acid substitutions are colored with respect to their localization in the HA protein: 120-loop of antigenic site in HA1 is colored in red, 150-loop – in green, 160-loop – in blue, 190-helix – in lilac. Amino acid positions with high Shannon entropy value are labeled in italics. Amino acids in the antigenic site surrounding RBS are colored in brown. Arrows indicate the period with fixed substitutions.

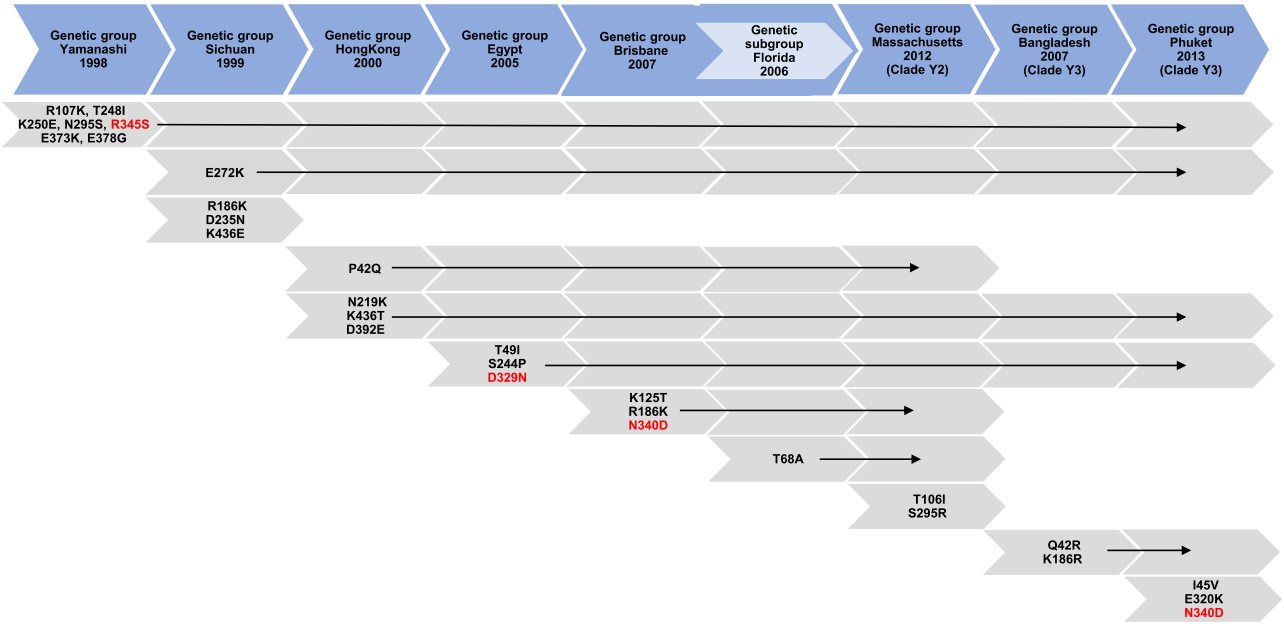

Fig. 6. Amino acid substitutions occurring along the phylogenetic 'trunk' and determining genetic group distinctions of NA of Yamagata-lineage viruses (compared with B/Beijing/184/1993). Amino acids in antigenic sites are colored in red. Arrows indicate the period with fixed substitutions.

The appearance of changes within the same antigenic region varied between 6 and 17 years. So, substitutions in the 120-loop and in the surrounding region occurred between 1996/1997 and 2013/2014, in the 150-loop in 1996/1997 and 2007/2008, in the 160-loop in 2001/2002 and 2007/2008, and the 190-helix between 1996/1997 and 2013/2014.

Changes at positions 198, 149, 126, 48, 162, 167, 182 and 150 were fixed since their first appearance whereas N165Y (in 2005/2006) and N202S (in 2011/2012) were seen only for one season, disappeared and were fixed two seasons later until now. A higher variability was evident for N116K that had viruses circulating between 1997 and 2002, then N116K disappeared for 9 years, re-appeared for one season and was then fixed again since 2014 (Fig. 9).

Some seasons were characterized by modification of an antigenic region alone or accompanied by fixed substitutions surrounding these neutralizing epitopes or RBS. Thus, the substitution G229D in the surrounding region of RBS appeared in 2005/2006 then was modified in some viruses by G229S and was fixed as G229D since 2013/2014 until now.

The substitutions described by Suptawiwat et al. (2017) as having a high Shannon entropy value at the positions 48 and 56 nearby the 120-loop (in addition to amino acids at antigenic sites) and at the position 40 outside of antigenic site have been fixed since 2004/2005. High Shannon entropy values were also described for amino acid position L172Q identified 2010/2011 outside of antigenic site as well as for K298E and E312K identified 2011/2012 (Fig. 9).

Other changes were temporary and seen for one or two seasons or a longer period (3 to 11 years) as N56T, T75I/V, V178H, G183E, V219I, V266I, I179V, P108A, T181A/K or V251M.

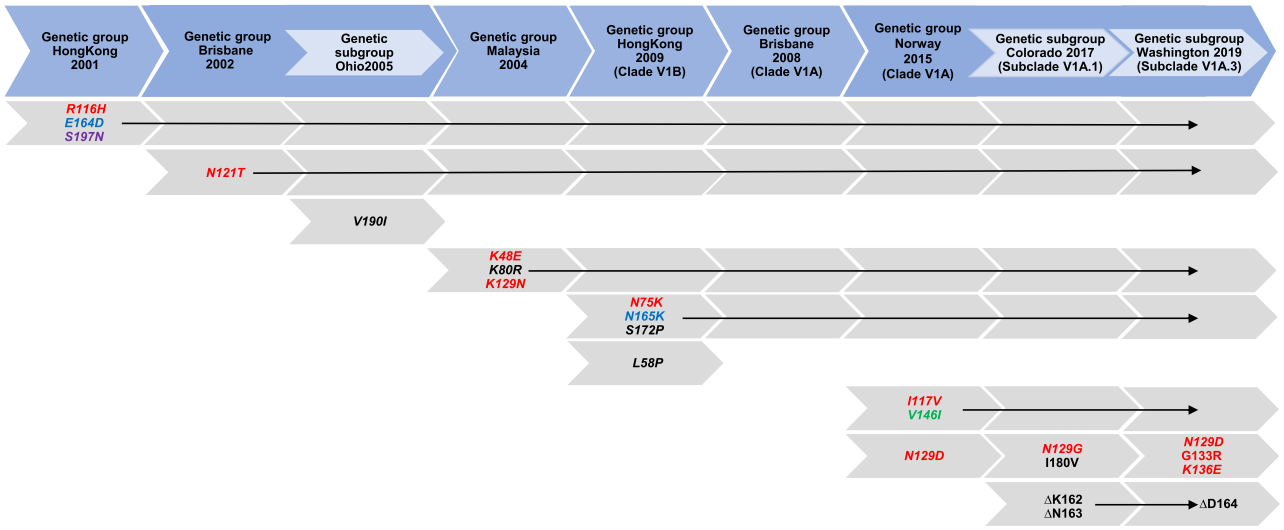

Fig. 7. Amino acid substitutions occurring along the phylogenetic 'trunk' and determining genetic group distinctions of HA1 of Victoria-lineage viruses (compared with B/Hong Kong/330/2001). Amino acid substitutions are colored with respect to their localization in the HA protein: 120-loop of antigenic site in HA1 is colored in red, 150-loop – in green, 160-loop – in blue, 190-helix – in lilac. Amino acid positions with high Shannon entropy value are labeled in italics. Arrows indicate the period with fixed substitutions.

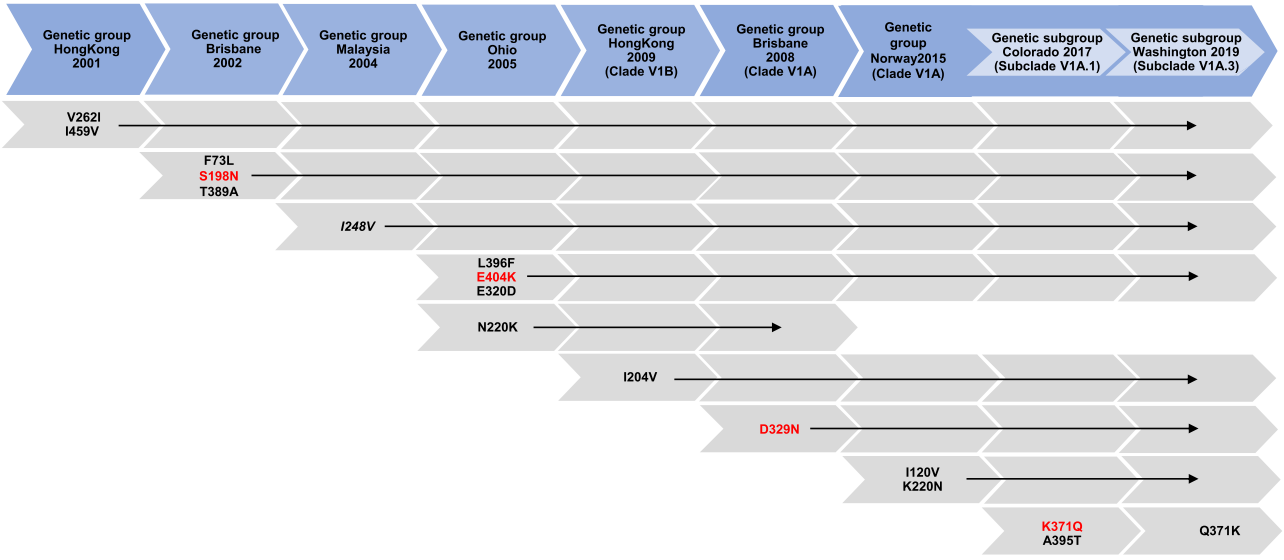

Fig. 8. Amino acid substitutions occurring along the phylogenetic 'trunk' and determining genetic group distinctions of NA of Victoria-lineage viruses (compared with B/Sichuan/379/1999). Amino acids in antigenic sites are colored in red. Arrows indicate the period with fixed substitutions.

3.3.2. *Victoria-lineage*

Altogether, thirteen substitutions were fixed over time, with eleven of them in the antigenic sites or close to it: five in the 120-loop (R116H, I117V, N121T, K129N/D, K136E) and two substitutions in the 120-loop surrounding region (K48E, N75K), one in the 150-loop (V146I), two in the 160-loop (E164D, N165K) and one in the 190-helix (S197N) (Fig. 10). The substitution G133R in the 120-loop has appeared for the first time in the season 2019/2020.

The appearance of fixed changes within the same antigenic region varied between 6 and 18 years. Substitutions in the 120-loop (or close to it) occurred between 2001/2002 and 2019/2020, in the 150-loop in 2008/2009 and 2014/2015, in the 160-loop in 2001/2002 and 2008/2009 and in the 190-helix in 2001/2002. Position 129 was replaced for the first time in the season 2005/2006 (K129N), modified by K129D in 2014/2015, then again by K129G in 2017/2018 and finally by K129D in 2018/2019 and preserved until now. Replacements V146I and V190I were typical only for one season. However, the substitution V190I appeared only in 2004/2005, while V146I appeared in the season 2008/2009, re-appeared in 2014/2015 and was fixed until now (Fig. 10).

Some seasons were characterized only by modification of an antigenic region alone or accompanied by substitutions surrounding these neutralizing epitopes or outside of antigenic site that were fixed since 2005/2006 (K48E, K80R) and since 2008/2009 (N75K, S172P) (Fig. 10). Positions 48 and 75 (located close to 120-loop) and positions 80 and 172 (outside of antigenic site) are known as residues with high Shannon entropy values (Suptawiwat et al., 2017). Other changes were temporary and seen only for one or two seasons. Substitutions close to the antigenic site as well as positions with high Shannon entropy values might have also contributed to appearance of new variants.

Deletions were not observed in Victoria-lineage viruses for years, but emerged recently. All viruses of the 2017/2018 season were characterized by a deletion of two amino acids at the position 162–163 of HA1 and thus formed the genetic subgroup Colorado2017 (represented by vaccine virus B/Colorado/06/2017; Δ162–163) within genetic group

Table 2
Genetic groups of influenza B viruses identified in Germany during influenza seasons 1996/1997 - 2019/2020.

	Genetic group	Influenza season*
HA Yamagata	Yamanashi1998	1997 + 1999
	Sichuan1999	1999 + 2000 + 2001 + 2002
	HongKong2000	2002
	Egypt2005	2005
	Florida2006	2007 + 2008
	Brisbane2007	2005 + 2006 + 2007 + 2008
	Massachusetts2012	2012 + 2013
	Bangladesh2007	2006 + 2008 + 2011 + 2012+2013
	Phuket2013	2012 + 2014–2019
NA Yamagata	Yamanashi 1998	1997 + 1999
	Sichuan1999	1999 + 2001 + 2002
	HongKong2000	2002
	Egypt2005	2005
	Brisbane2007	2005 + 2006 + 2008
	Florida2006 (genetic subgroup within genetic group Brisbane 2007)	2007 + 2008
	Massachusetts2012	2012 + 2013
	Bangladesh2007	2006 + 2008 + 2011 + 2012 + 2013
	Phuket2013	2014 – 2019
HA Victoria	HongKong2001	2002
	Brisbane2002	2002 + 2003
	Ohio2005 (genetic subgroup within genetic group Brisbane 2002)	2005
	Malaysia2004	2006 + 2007 + 2008+2011
	HongKong2009	2010+ 2011 + 2012
	Brisbane2008	2009 + 2010 + 2011 + 2012 + 2013 + 2014
	Norway2015	2015 + 2016 + 2017
	Colorado2017 (genetic subgroup within genetic group Norway2015)	2018
	Washington2019 (genetic subgroup within genetic group Norway2015)	2019 + 2020
NA Victoria	HongKong2001	2002
	Brisbane2002	2002 + 2003 + 2011 + 2012
	Malaysia2004	2003 + 2005
	Ohio2005	2006 + 2007 + 2012
	HongKong2009	2011
	Brisbane2008	2009 + 2011 + 2012 + 2013 + 2014
	Norway2015	2015 + 2016 + 2017
	Colorado2017 (genetic subgroup within genetic group Norway2015)	2018
	Washington2019 (genetic subgroup within genetic group Norway2015)	2019 + 2020

* influenza seasons are indicated by the last year of the circulation of seasonal viruses (e.g. the season 1996/1997 is presented as 1997).

Norway2015. Viruses with triple deletion Δ162–164 were found in Germany since season 2018/2019. These viruses formed the genetic subgroup Washington2019 represented by vaccine virus B/Washington/02/2019. The protruding nature of the 160-loop may make it easy to accommodate even multiple-residue insertions or deletions (Wang et al., 2008).

3.4. Molecular characterization of neuraminidase gene of influenza B viruses

3.4.1. Yamagata-lineage

Altogether, twenty-two substitutions were fixed over time. Yamagata-lineage viruses circulating in Germany since 1997 acquired a number of substitutions both in the globular domain and in the stalk region of NA (Fig. 11). The viruses identified in the season 1996/1997 possessed the substitutions R107K, R345S, N295S and E373K in known antigenic sites of influenza B neuraminidase (Air et al., 1990; Burmeister et al., 1992) as well as substitutions K250E, E378G and T248I compared to vaccine virus B/Beijing/184/1993. All viruses circulating since 1996/1997 retained these substitutions. An exception is the substitution at position 373. Only some viruses with Q373 were identified first in 2015/2016 whereas all viruses circulating since 2017/2018 possessed Q373.

The substitution N295R appeared first in 2007/2008 and re-appeared in 2011/2012 and was characteristic for the most viruses circulating until 2016/2017 (Fig. 11). All viruses circulating since 2017/2018 possessed at this position S (serine) again. Similarly, the substitution T248V appeared first in 2011/2012 and some viruses possessed this substitution until 2013/2014. All viruses isolated since 2014/2015 had in this position I (isoleucine) again.

The viruses circulating in the subsequent seasons obtained some additional substitutions (Fig. 11). Most newly acquired substitutions have so far been preserved. Exceptions were six substitutions appearing in NA in 1998/1999 (K436E, L73F, D342N, R186K, E148G, D235N), the substitution P42Q appearing in 2001/2002, two substitutions appearing in 2004/2005 (T49I and K125T) and finally, the substitution T106I appearing in 2011/2012.

3.4.2. Victoria-lineage

Altogether, seventeen substitutions were fixed over time. Victoria-lineage viruses detected in Germany since 2002 were reassortants with a Victoria-like HA and a Yamagata-lineage NA gene. The viruses circulating in Germany during 2001/2002 possessed the substitutions E148G and S198N in known antigenic sites of influenza B neuraminidase as well as the substitutions F73L, I248V and T389A compared to vaccine virus B/Sichuan/379/1999. The viruses circulating in the subsequent seasons obtained some additional substitutions (Fig. 12). All these newly acquired substitutions have so far been preserved. Exceptions were two substitutions appearing in NA in 2004/2005 (E404G) and in 2008/2009 (A358E) that were characteristic of viruses circulating until 2005/2006

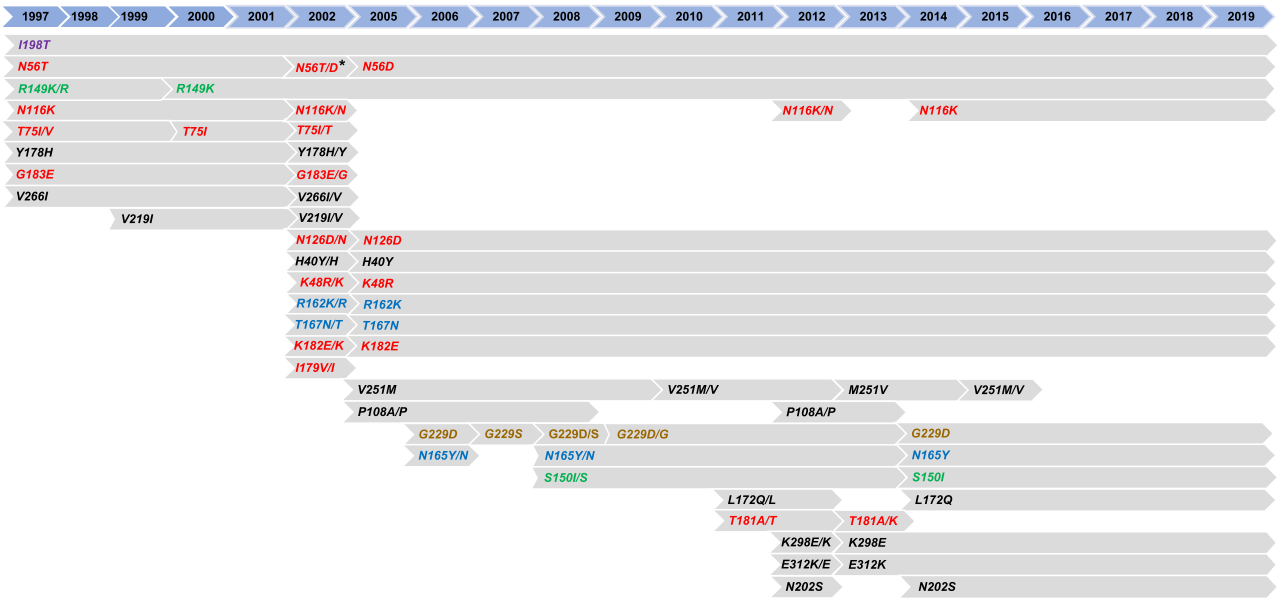

Fig. 9. Amino acid substitutions in the HA1 region of influenza B Yamagata-lineage viruses identified in Germany during 1996/1997–2018/2019 (lines marked in gray) compared to vaccine virus B/Beijing/184/1993. Amino acid substitutions are colored with respect to their localization in the HA protein: 120-loop of antigenic site in HA1 is colored in red, 150-loop – in green, 160-loop – in blue, 190-helix – in lilac. Amino acids in the antigenic site surrounding RBS are colored in brown. Amino acid positions with high Shannon entropy value are labeled in italics. Influenza seasons are indicated by the last year of the circulation of seasonal viruses. *Spelling example: N56T/D – co-circulation of two virus variants with amino acid T or D at the position 56.

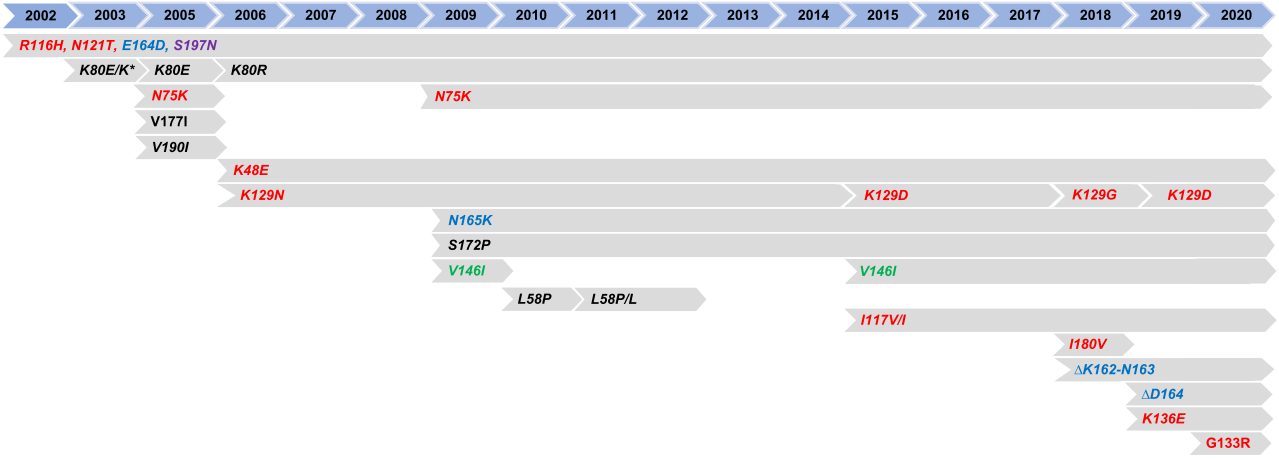

Fig. 10. Amino acid substitutions in the HA1 region of influenza B viruses of Victoria-lineage identified in Germany during 2001/2002–2019/2020 (lines marked in gray) compared to vaccine virus B/Hong Kong/330/2001. Amino acid substitutions are colored with respect to their localization in the HA protein: 120-loop of antigenic site in HA1 is colored in red, 150-loop – in green, 160-loop – in blue, 190-helix – in lilac. Amino acid positions with high Shannon entropy value are labeled in italics. Influenza seasons are indicated by the last year of the circulation of seasonal viruses. *Spelling example: K80E/K – co-circulation of two virus variants with amino acid E or K at the position 80.

and 2011/2012, respectively. Further, exceptions were four substitutions at positions 51, 220, 371 and 395 of NA. The substitution N220K appeared in 2005/2006 and was characteristic of viruses circulating only until 2015/2016. P51S/L was seen only in the 2010/2011 season, and the substitutions K371Q and A395V were typical for viruses circulating during 2017/2018.

3.5. Occurrence of reassortment in several influenza B viruses

Analyses of both the HA and NA segments together revealed five inter-lineage, twenty-seven intra-clade (inter-genetic) and one inter-clade reassortants that were isolated during the time period 1996/1997–2019/2020.

3.5.1. Inter-lineage reassortants

Five influenza B viruses isolated in Germany in 2002, 2012 and 2015 were found to be reassortants between HA and NA genes of the Yamagata- and Victoria- lineages.

Reassortants B/MVP/12/2002 and B/BWB/15/2012 possessed the Victoria-like HA gene and the Yamagata-like NA gene. The virus B/MVP/12/2002 had the HA gene that was closely related to that of the vaccine virus B/Hong Kong/330/2001 and was characterized by B/Sichuan/379/1999-like NA gene, whereas the second virus possessed the HA gene closely related to that of the reference virus B/Malta/MV636714/2011 (genetic group Brisbane2008 within clade V.1A of Victoria-lineage), and the NA gene closely related to the reference virus B/Stockholm/12/2011 (Bangladesh2007 genetic group within clade Y3 of Yamagata-lineage).

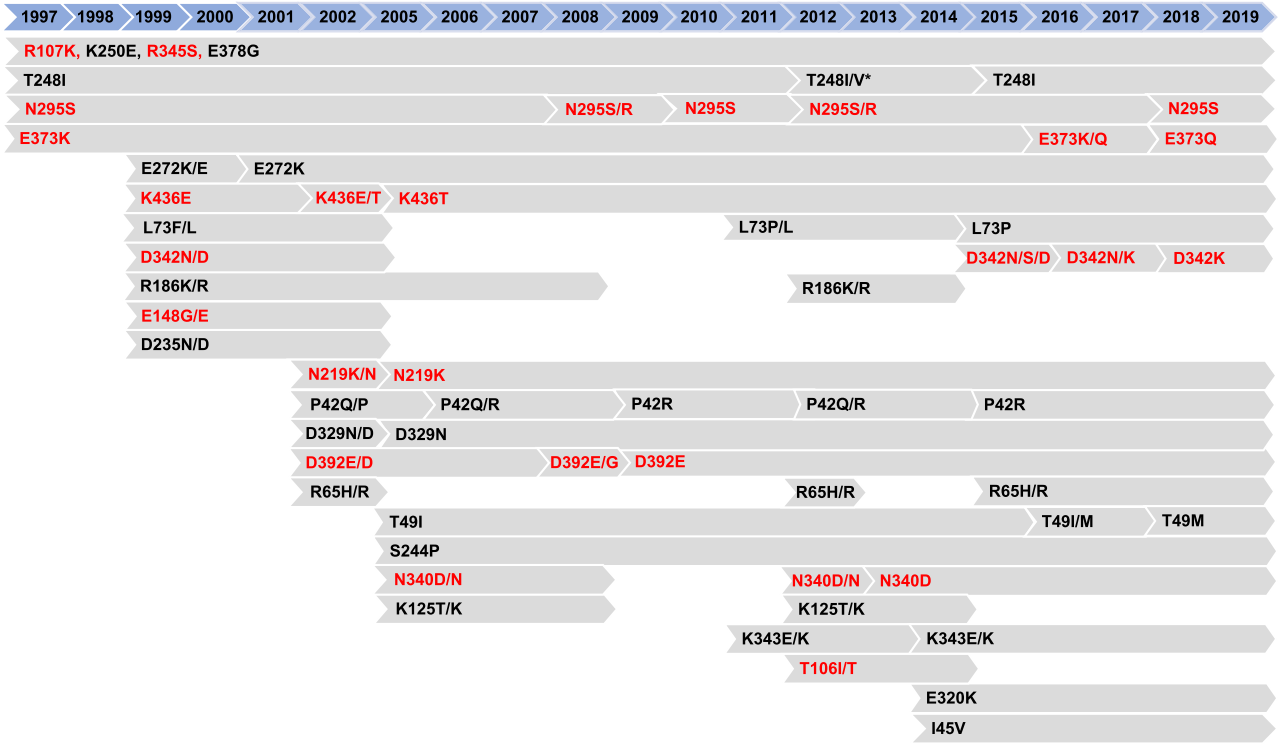

Fig. 11. Amino acid substitutions in the NA gene of influenza B viruses of Yamagata-lineage identified in Germany during 1996/1997–2018/2019 (lines marked in gray) compared to vaccine virus B/Beijing/184/1993. Amino acid substitutions in the antigenic site are colored in red. Influenza seasons are indicated by the last year of the circulation of seasonal viruses. *Spelling example: T248I/V – co-circulation of two virus variants with amino acid I or V at the position 248.

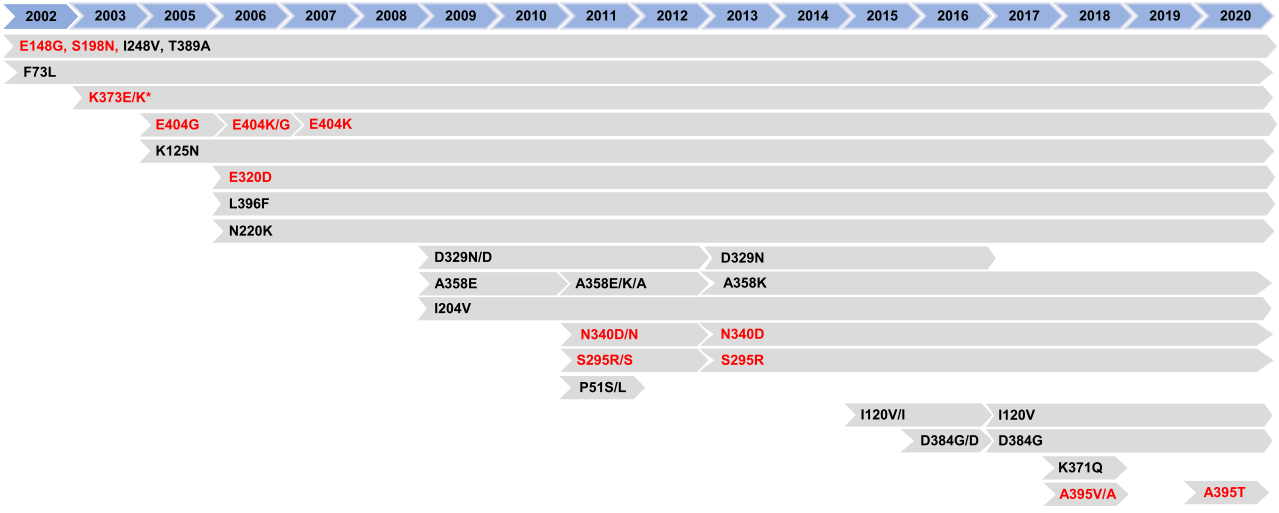

Fig. 12. Amino acid substitutions in the NA gene of influenza B viruses of Victoria-lineage identified in Germany during 2001/2002–2019/2020 (lines marked in gray) compared to vaccine virus B/Sichuan/379/1999. Amino acid substitutions in the antigenic site are colored in red. Influenza seasons are indicated by the last year of the circulation of season viruses. *Spelling example: K373E/K – co-circulation of two virus variants with amino acid E or K at the position 373.

On the contrary, the reassortants B/BWB/66/2002, B/BLN/6/2012 and B/HES/15/2015 had Yamagata-like HA genes, namely, B/Sichuan/379/1999-like (genetic group Sichuan1999), B/Stockholm/12/2011-like (Bangladesh2007 genetic group) and B/Wisconsin/1/2010-like (genetic group Phuket2013), respectively. The NA genes of these viruses represented the Victoria-lineage. The first reassortant was characterized by the NA gene closely related to that of the vaccine virus B/Hong Kong/330/2001 whereas the second and third one had the NA gene that was close to that of the reference virus B/Malta/MV636714/2011 and B/Johannesburg/3964/2012 (both belong to the genetic group Brisbane2008 within clade V.1A), respectively.

3.5.2. *Intra-clade reassortants*

Reassortants of the Yamagata-lineage as B/BWB/25/2002, B/NRW/14/2002 and B/HES/28/2002 possessed the HA genes that belonged to the genetic group Hong Kong2000 whereas the NA genes represented the genetic group Sichuan1999. On the contrary, B/NSA/36/2002 and B/BWB/26/2002 (both of the Yamagata-lineage) possessed the HA genes that belonged to the genetic group Sichuan1999 whereas the NA genes of these viruses belonged to the Hong Kong2000 group.

The virus B/BLN/4/2012 was found to be an intra-clade reassortant within the Victoria- lineage. This virus possessed the HA gene closely related to that of the reference virus B/Hong Kong/514/2009 (genetic group HongKong2009, clade V.1B), whereas the NA gene of this virus was close to that of the vaccine virus B/Malaysia/250/2004 (genetic group Malaysia2004).

Further on, we also revealed that HA and NA sequences of five Victoria-like viruses identified in the season 2004/2005 (B/BAY/22/2005, B/BLN/5/2005, B/SAT/6/2005, B/THR/12/2005, B/THR14/2005) belonged to different genetic groups, namely, HA to the genetic subgroup Ohio2005 (within genetic group Brisbane2002) and NA to the genetic group Malaysia2004.

On the contrary, NA of the 15 viruses circulating in 2005/2006 and one virus of the 2006/2007 season (B/BWB5/2007) represented the genetic group Ohio2005 while HA of these viruses belonged to genetic group Malaysia2004.

In addition, we support that two vaccine viruses B/Shanghai/361/2002 and B/Jiangsu/10/2003 are intra-clade reassortants: the HA gene of these viruses belongs to the genetic group HongKong2000 whereas the NA gene - to the genetic group Sichuan1999, both groups within clade Y2 of Yamagata-lineage.

3.5.3. *Inter-clade reassortants*

The virus B/BAY/14/2012 was found to be an inter-clade reassortant within Victoria-lineage. The HA gene of this virus belonged to the genetic group Brisbane 2008 (clade V.1A) whereas the NA gene represented the genetic group Ohio2005.

3.6. *Evolutionary rates of influenza B viruses*

To estimate the time of natural evolution, we estimated the rates of nucleotide substitution for both type B lineages.

Estimated rate of nucleotide substitution of HA of the Victoria-lineage (mean clock rate 2.05×10^{-3} substitution/site/year, s/s/y) was lower than that for HA of the Yamagata-lineage (mean clock rate 2.88×10^{-3} s/s/y). Similarly, rate of nucleotide substitution of NA of the Victoria-lineage (mean clock rate 2.01×10^{-3} s/s/y) was lower than that estimated for NA of the Yamagata-lineage (mean clock rate 2.35×10^{-3} s/s/y).

4. Discussion

Different patterns of dominant type of influenza virus were observed across Germany over the study period. For example, influenza B viruses made up the majority (68%) of viruses circulating in Germany in 2017/2018 while influenza A viruses dominated in other countries (USA, Canada, United Kingdom, Slovenia (FluNet). During this season, the prevalence of both type B lineages varied in different countries. In Germany, B/Yamagata-lineage viruses prevailed over B/Victoria-lineage at ratio of 99:1 while in USA at ratio of 8:1 and in Europe at ratio of 50:1 was identified (ECDC 2018; ECDC/WHO 2018; FluNet; Garten et al., 2018). Thus, a mismatch with the trivalent vaccine recommended by the WHO that included the B/Victoria-lineage virus was observed in Germany in this influenza season.

Differing lineage prevalence between Germany and most other European countries was also seen in 2004/2005. The majority of circulating B viruses belonged to the Yamagata-lineage whereas B/Victoria-lineage viruses dominated in Germany at the ratio of 3:1 (WHO 2005a, 2005b). According to our data, it would have been optimal to choose a B/Victoria-like virus as vaccine strain for 2005/2006. Indeed, B/Victoria viruses predominated in most countries in the next season. However, trivalent vaccines for 2005/2006 - as well as in the season 2004/2005 - included the B/Yamagata-lineage virus B/Shanghai/361/2002 at that time. Thus, influenza B viruses circulating in Germany during those seasons had lineage mismatch with the trivalent vaccine recommended by the WHO (WHO 2005b, 2006a, 2006b).

Numerous studies have shown that vaccination against one lineage results in little cross-protection from the other and was estimated at 30% (Piralla et al. 2017; Skowronski et al. 2014). Therefore, it was suggested to include viruses of both influenza B lineages to improve effectiveness (Belshe 2010; Ray et al., 2017; Tewawong et al., 2015; Tisa et al., 2016). In February 2013, the WHO issued its first guidelines recommending to including both lineages in the vaccine composition (WHO 2013). In Germany, since 2018, the vaccination has been carried out using quadrivalent vaccines (STIKO 2018).

We have followed the evolutionary dynamics of influenza B viruses circulating in Germany over the past 25 years by studying the pattern of changes in the HA and NA gene based on sequences established by Sanger sequencing. However, it should be noted that due to error-prone replication and high mutation rates, RNA viral populations consist of mutant spectra and viruses exist in infected hosts as “quasispecies”. In virology, quasispecies is defined as the set of mutant genomes that comprise viral populations (Domingo et al., 2021). Many events in viral pathogenesis and evolution are due to mutant spectrum modifications or interactions (Domingo et al., 2012). Only using of deep sequencing as NGS is able to identify low-frequency mutations (i.e. <20%) and characterize quasispecies (Domingo and Perales 2019; Knyazev et al., 2021). A mutant spectrum defines a consensus which is calculated of most frequent nucleotide, found at each position in a sequence alignment. It serves as a simplified representation of the viral population (Domingo et al., 2021).

Our phylogenetic analyses showed that Yamagata-lineage viruses are more diverse than Victoria-lineage viruses. This allows the B/Yamagata viruses to evolve forming different genetic groups in a paraphyletic or polyphyletic fashion. For example, the viruses of six seasons (1998/1999, 2001/2002, 2004/2005, 2005/2006, 2006/2007 and 2012/2013) were each found in two different genetic groups and those of two other seasons (2007/2008, 2011/2012) were detected even in three genetic groups. This demonstrated that B/Yamagata viruses of different genetic groups could emerge and co-circulate simultaneously. Yamagata-lineage viruses of each genetic group circulated usually from one to seven seasons. So, viruses of the genetic group Florida2006 were identified in Germany only in 2006/2007 and 2007/2008, while Florida2006 group viruses circulated in other countries (Japan, Bolivia, Taiwan) also during 2008/2009 (WHO 2009). However, viruses of the Phuket2013 group have been circulating in Germany and worldwide since 2014 (Kato-Miyashita et al. 2020; WHO 2013; WHO 2014; WHO 2019). The vaccine virus B/Phuket/3073/2013 representing the Yamagata-lineage remained unchanged since 2014 consistent with the lack of trunk amino acid substitutions in HA of the viruses isolated during this time period.

In contrast, the Victoria-lineage viruses were more homogeneous. Only viruses of four seasons were distributed in different genetic groups: viruses of 2001/2002, 2019/2010 and 2011/2012 belonged to two genetic groups and the viruses of 2010/2011 were distributed between three genetic groups. Victoria-lineage viruses of each genetic group circulated from one to six seasons. Thus, the viruses of six seasons compose the genetic group Brisbane2008 (clade V.1A). This is in line with previously published studies (Kuo et al., 2016; Langat et al. 2017).

The results of comparative phylogenetic analysis of both Yamagata- and Victoria-lineages of influenza B viruses circulating in Germany are in good agreement with our data on the rate of nucleotide substitution in both influenza B lineages. Estimated molecular clock rates of nucleotide substitution of Victoria-lineage HA averaged 2.05×10^{-3} and of Yamagata-lineage HA averaged 2.88×10^{-3} s/s/y. These rates were considerably lower than those of A(H3N2) and A(H1N1) human influenza viruses with 5.0×10^{-3} and 4.4×10^{-3} s/s/y, respectively (Bedford et al. 2015; Rambaut et al., 2008), and that consistent with previous work (Nelson et al. 2006). On the other hand, our results on molecular clock analyses are in contrast with some data reported earlier. So, estimate rates of nucleotide substitution of Yamagata-lineage HA were

lower than those of Victoria-lineage HA with 1.32×10^{-3} and 3.32×10^{-3} as well as with 2.01×10^{-3} and 2.16×10^{-3} and 2.00×10^{-3} and 2.43×10^{-3} s/s/y, respectively (Chen and Holmes 2008; Vijaykrishna et al. 2015; Yoshihara et al. 2020). Besides, the recent analysis with Bayesian evolutionary model using global dataset showed that Victoria and Yamagata lineages have comparable rates of antigenic drift (Langat et al. 2017). However, our data are in good agreement with other studies. Estimate rates of nucleotide substitution of HA of Victoria-lineage were lower than those of Yamagata-lineage with 2.7×10^{-3} and 3.8×10^{-3} s/s/y, respectively (Pechirra et al. 2005). In line with our results is also the report published in 2020 (Virk et al. 2020) with nucleotide substitution rates that were lower for HA and NA of Victoria lineage viruses (1.93×10^{-3} and $2.41 \times 10^{-3\ s/s/y}$) than for HA and NA of Yamagata lineage viruses (2.75×10^{-3} and $2.82 \times 10^{-3\ s/s/y}$). The difference in the rate of evolution between studies could be explained by the difference in geographic location and the year of the study (Langat et al. 2017). In addition, the vaccine coverage and current vaccine formulation as one of an evolution driving force cannot be eliminated (Lam et al., 2017).

Phylogenetic analyses of HA nucleotide sequences and deduced amino acids described here revealed a number of significant amino acid substitutions in four major epitopes of HA (120-loop, 150-loop, 160-loop and 190-helix) and in the 120-loop surrounding region (residues 48, 56, 58, 75, 179, 180, 181, 182). We observed that most of the amino acid substitutions were located in the 120-loop of both influenza B virus lineages suggesting that this site is the most frequently altered HA region confirming the findings of other studies (Langat et al. 2017; Wang et al., 2008). We have also determined some replacements in the 190-helix of the RBS and in the region surrounding the RBS. The structure of the antigenic site, RBS and the surrounding region is regarded as an important factor for efficient binding of antibodies and receptors (Wang et al., 2008). Furthermore, we found a number of substitutions outside of antigenic site at the amino acid positions that are known to have increased Shannon entropy and could contribute to the antigenic properties of viruses (Suptawiwat et al., 2017). Besides, some replacements led to the emergence of potential glycosylation sites and, thus, may also contribute to antigenic drift (Wang et al., 2008).

The predominance of the amino acid changes on the top of the NA suggests that this is the position of antigenic sites (Air et al., 1990; Burmeister et al., 1992; Colman et al., 1983). Thus, many substitutions that we detected in Yamagata- (R107K, R345S, N295S/R, E373K/Q, K436E/T, D342N/S/K, E148G, N219K, D392E/G, N340D, T106I) and Victoria-lineage viruses (E148G, S198N, K373E, E404G/K, E320D, N340D, S295R, A395V/T) were located in the antigenic sites of the NA. This indicates that immune selection is not only a basic mechanism for HA but also for NA evolution. These results are in line with recently reported data suggesting that the NA is contributing significantly to the evolution of influenza B virus (Virk et al. 2020). Besides, the substitutions in the stalk region of NA might contribute to the evolution of influenza B virus by changing their properties. Others have also demonstrated that a single mutation in the stalk can significantly affect enzyme activity, presumably affecting the stability of the tetramer (Zanin et al. 2017). There are several important conserved residues in the NA active site of influenza B virus which interacts with sialic acids and several conserved residues which do not contact sialic acids, but have important structural role and are defined as frameworks (Colman et al., 1983; McAuley et al., 2019). Some authors suggest that D197 belongs to the framework but is not conserved (Burmeister et al., 1992). None of the NA gene sequences investigated displayed substitutions in the active center and their surrounding residues except one sequence that had D197N substitution (B/NRW/35/2018).

Segment reassortment between the Victoria- and Yamagata-lineages that based on analysis of the HA and NA genes has been reported since 1999 (Matsuzaki et al. 2004; McCullers and Hayden 2012; McCullers et al., 1999; Shaw et al. 2002). Reassortment of surface protein genes enabled a significant increase in genetic diversity in addition to natural mutations (Matsuzaki et al. 2004). Reassortants with a Victoria-like HA and a Yamagata-like NA gene were detected in Germany for the first time during 2001/2002 but circulated also worldwide (Biere and Schweiger 2008; Shaw et al. 2002). Genetic grouping allowed the classification of inter-lineage, inter-clade or intra-clade reassortants. We identified five inter-lineage, one inter-clade and twenty-seven intra--clade reassortants. This finding is in line with other reports indicating that genetic reassortment occurred not only between viruses belonging to different lineages, but also between viruses belonging to separate clades (Kuo et al., 2016; Matsuzaki et al. 2004; Oong et al. 2017; Puzelli et al. 2004). Thus, our findings along with data from others support the idea that multiple reassortment process allows influenza B virus to escape host immunity and continue to evolve without formation of subtypes (McCullers et al., 1999).

In this study, we focused also on features that might be more specific to our country including the appearance of herald viruses and new virus variants predominating during the next season. Herald viruses were considered to be the parental viruses for the following influenza season that might have full or partial genetic signature compared to the following epidemic viruses. The first report on the influenza B virus herald wave in early 1976, one year before this virus became epidemic, was published in 1980 (Glezen et al., 1980). Later, herald strains of influenza B viruses were isolated in Japan in 1987 and 1989 predominating the next winter season (Moriuchi et al., 1991; Nakajima et al., 1992).

In 2006, the appearance and accumulation of substitutions N165Y and G229D in Yamagata-lineage viruses potentially affected antigenicity and led to formation of the new Bangladesh2007 group (clade Y3). Two viruses isolated in Germany in 2006 possessed the clade Y3-specific substitutions N165Y and G229D while all the other viruses of this season did not. Thus, these two viruses are regarded as herald strains and were the first clade 3 viruses identified in Germany. Two years later, clade Y3 viruses accounted for 36% (9 of 25 isolated viruses) of Yamagata-lineage viruses identified in Germany during 2007/2008. Notably, two viruses – one from Canada and one from Honduras - isolated even earlier, in November and December 2005, respectively, had also the additional changes N165Y and G229D. Some viruses with these substitutions were also identified in Finland, Norway and Sweden in early 2006 (WHO 2006a). In Germany, other herald viruses were also identified in 2011/2012. They possessed N116K and N202S substitutions that were fixed two years later leading to formation of the new genetic group Phuket2013 that differed also antigenically from the Bangladesh2007group. Such viruses from herald waves of influenza could be used for vaccine development and, thus, their early detection is essential for prediction of the virus that will be dominant in the following influenza season (Alfaro-Murillo et al., 2013).

The findings presented here show evolutionary changes within and between two influenza B lineages. They are most probably the results of changes in herd immunity and its evolutionary pressure. It should not be ruled out that herd immunity in different countries/continents is different and has its own characteristics and, hence, different countries might have different evolutionary dynamics of influenza B viruses.

5. Conclusion

Our long-term study showed evolution features for influenza B viruses circulating in Germany that were comparable with those reported from other countries. However, we identified also viruses and circulation patterns that were more specific for our country such as lineage prevalence and genetic features of lineages, some reassortment events or detection of herald viruses.

We determined key amino acid substitutions in the antigenic epitopes of HA and replacements in the globular domain of NA that may contribute to the epidemic potential of influenza B viruses circulating in Germany by leading to the spread of new genetic groups with an antigenic profile that may differ significantly from that of the current

vaccine.

Phylogenetic analysis of HA and NA segments together revealed some inter-lineage, inter- and intra-clade reassortants supporting the assumption that reassortment process is an important evolution mechanism generating new influenza B viruses with optimal match of HA and NA features.

In summary, the data obtained underscore the importance of continuous monitoring of circulating influenza B viruses to provide early identification of strains with genetic and antigenic variations to predict the strains for the following season. The knowledge about circulating viruses is essential for an optimal vaccine composition and the management of influenza in the respective region.

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CRediT authorship contribution statement

Alla Heider: Conceptualization, Investigation, Formal analysis, Writing – original draft. **Marianne Wedde:** Investigation, Formal analysis, Writing – review & editing. **Ralf Dürrwald:** Supervision, Writing – review & editing. **Thorsten Wolff:** Supervision, Writing – review & editing. **Brunhilde Schweiger:** Conceptualization, Supervision, Writing – review & editing.

Declaration of Competing Interest

The authors report no declarations of interest.

Data Availability

Data will be made available on request.

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Supplementary materials

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