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Substitutions near the HA receptor binding site explain the origin and major antigenic change of the B/Victoria and B/Yamagata lineages

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Influenza B virus primarily infects humans, causing seasonal epidemics globally. Two antigenic variants—Victoria-like and Yamagata-like—were detected in the 1980s, of which the molecular basis of emergence is still incompletely understood. Here, the antigenic properties of a unique collection of historical virus isolates, sampled from 1962 to 2000 and passaged exclusively in mammalian cells to preserve antigenic properties, were determined with the hemagglutination inhibition assay and an antigenic map was built to quantify and visualize the divergence of the lineages. The antigenic map revealed only three distinct antigenic clusters—Early, Victoria, and Yamagata—with relatively little antigenic diversity in each cluster until 2000. Viruses with Victoria-like antigenic properties emerged around 1972 and diversified subsequently into two genetic lineages. Viruses with Yamagata-like antigenic properties evolved from one lineage and became clearly antigenically distinct from the Victoria-like viruses around 1988. Recombinant mutant viruses were tested to show that insertions and deletions (indels), as observed frequently in influenza B virus hemagglutinin, had little effect on antigenic properties. In contrast, amino-acid substitutions at positions 148, 149, 150, and 203, adjacent to the hemagglutinin receptor binding site, determined the main antigenic differences between the Early, Victoria-like, and Yamagata-like viruses. Surprisingly, substitutions at two of the four positions reverted in recent viruses of the Victoria lineage, resulting in antigenic properties similar to viruses circulating ∼50 y earlier. These data shed light on the antigenic diversification of influenza viruses and suggest there may be limits to the antigenic evolution of influenza B virus.

influenza B virus | antigenic evolution | vaccines | hemagglutinin

Significance

Influenza viruses are estimated to infect up to 20% of people during annual epidemics, causing substantial morbidity and mortality, particularly among risk groups. Vaccines continue to be the best available preventive measure, but their efficacy is hindered by constant viral immune evasion. Two cocirculating influenza B virus lineages, B/Victoria and B/Yamagata, are antigenically distinct, and immunity against one lineage does not protect optimally against infection with the other. By investigating the genetic and antigenic data of a historical influenza B virus collection, we reconstructed the antigenic evolution and identified the molecular determinants of the early antigenic diversification. This research improves our understanding of the emergence of new antigenic variants of influenza virus, important for influenza vaccine design and strain selection.

Influenza A and B viruses cause seasonal epidemics in humans and pose a major challenge to public health. Although influenza B virus is frequently considered a milder virus compared with influenza A virus, influenza B virus infections contribute substantially to the estimated 290,000–650,000 annual influenza-related global deaths and are the prominent circulating subtype of influenza every 4 to 5 y (1).

Influenza B virus was first isolated in the 1940s (2). Shortly thereafter, an influenza B virus component was included in bivalent influenza vaccines that continued to be updated since. During the 1988–1989 season, two distinct cocirculating antigenic variants of influenza B virus were recognized, the B/Victoria and B/Yamagata lineages, named after the reference strains B/Victoria/2/87 and B/Yamagata/16/88, respectively (3). It is currently thought that the two lineages diverged genetically in the 1970s (4, 5). The B/Victoria lineage circulated widely from the mid to late 1980s, while the B/Yamagata lineage predominated in the 1990s (6), a decade in which B/Victoria was largely confined to China (7, 8). Since 2000, the B/Victoria lineage re-emerged globally and both influenza B virus lineages have cocirculated in the Northern and Southern Hemispheres (9, 10). This repeated cocirculation resulted in the development of quadrivalent influenza vaccines that protect against both lineages, as opposed to trivalent vaccines that contained only one of the lineages and have led to vaccine mismatches, potentially associated with low vaccine efficacy (11).

The evolutionary dynamics of influenza B virus are complex and have been characterized by nucleotide insertions and deletions (indels) in the hemagglutinin (HA) gene and extensive reassortment events within and between the B/Victoria and B/Yamagata lineages (12, 13). Remarkably, only the polymerase gene segments PB2 and PB1 along with HA formed separate lineages over time, while other segments have continuously reassorted between lineages (14). Antigenic drift is another important mechanism that contributes to the diversification of influenza B viruses through accumulation of amino-acid substitutions in HA that result in virus escape from pre-existing immunity in the population (15–18). The antigenic characteristics of influenza viruses are routinely

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assessed with the hemagglutination inhibition (HI) assay by measuring the cross-reactivity of viruses with antisera. The rate of antigenic change observed for influenza B virus was shown to be slower than for influenza A viruses (19), as well as its nucleotide substitution rate (20–22). Studies involving site-directed mutagenesis, monoclonal antibody (mAb)-selected escape mutants, or field isolates have helped determine four major antigenic sites involved in the antigenic evolution of influenza B HA: the 120 loop, the 150 loop, the 160 loop, the 190 helix, and their surrounding regions (15, 18, 22–26). Together, they are thought to form a more or less continuous antigenic space (27). The 120 loop partially overlaps with site E, the 150 loop with site A, and the 160 loop and 190 helix with site B on the H3 HA structure (28). Indels have been identified frequently in the 160 loop in field isolates and in mAb escape mutants (7, 12, 25), but their contribution to antigenic drift is not fully understood.

High levels of epidemic activity of the influenza B viruses coupled with an increase in genetic diversity in the B/Victoria lineage, which has recently experienced extensive diversification into cocirculating clades (13), may pose a future challenge to the existing quadrivalent vaccines. The lack of detection of viruses of the B/Yamagata lineage in recent years, on the other hand, may reduce the complexity of vaccine strain selection. In order to better understand the emergence of new antigenic variants of influenza B virus and inform the development of surveillance systems and vaccine selection, we reinvestigated the emergence of the B/Victoria and B/Yamagata lineages by exploring both the genetic and antigenic evolution of early viruses. To this end, a unique collection of virus isolates spanning a period of more than 3 decades that were exclusively passaged in mammalian cells to ensure preservation of the genetic and antigenic properties of the original isolates was used to generate an antigenic map, along with vaccine and other reference strains. Major antigenic changes associated with the emergence of the Victoria-like and Yamagata-like viruses were mapped to the 150 loop and 190 helix, while indels did not play a direct role. Changes at these positions again were important for antigenic variants of the B/Victoria lineage that emerged in 2019. This work advances our understanding of the molecular changes that shape the antigenic evolution of influenza B virus and provides insights for the annual vaccine strain selection process.

Results

A Historical Collection of Cell Culture Influenza B Virus Isolates. A set of 45 influenza B viruses, sampled in the Netherlands from 1962 until 1998, was selected from the Erasmus Medical Centre (MC) biobank. These viruses were passaged exclusively in cell culture, for three to six passages, to minimize genetic and antigenic changes known to be associated with propagation in embryonated eggs. The HA genes of these virus isolates were sequenced by Sanger sequencing (*SI Appendix*, Table S1), and this sequence dataset was compared with all publicly available HA1 sequences of influenza B viruses isolated worldwide between 1940 and 2000. A maximum likelihood (ML) phylogenetic analysis of this total set of 617 HA1 sequences revealed that the Dutch virus collection represented the global diversity of influenza B viruses well, with all major genetic lineages across the phylogenetic tree containing one or more cell culture virus isolates (Fig. 1). Specifically, seven virus isolates belonged to the lineage evolving from the B/Victoria/2/1987-like viruses, 15 to the lineage evolving from the B/Yamagata/16/1988-like viruses and 23 were viruses circulating from 1962 until the emergence of the B/Victoria-like and B/Yamagata-like viruses.

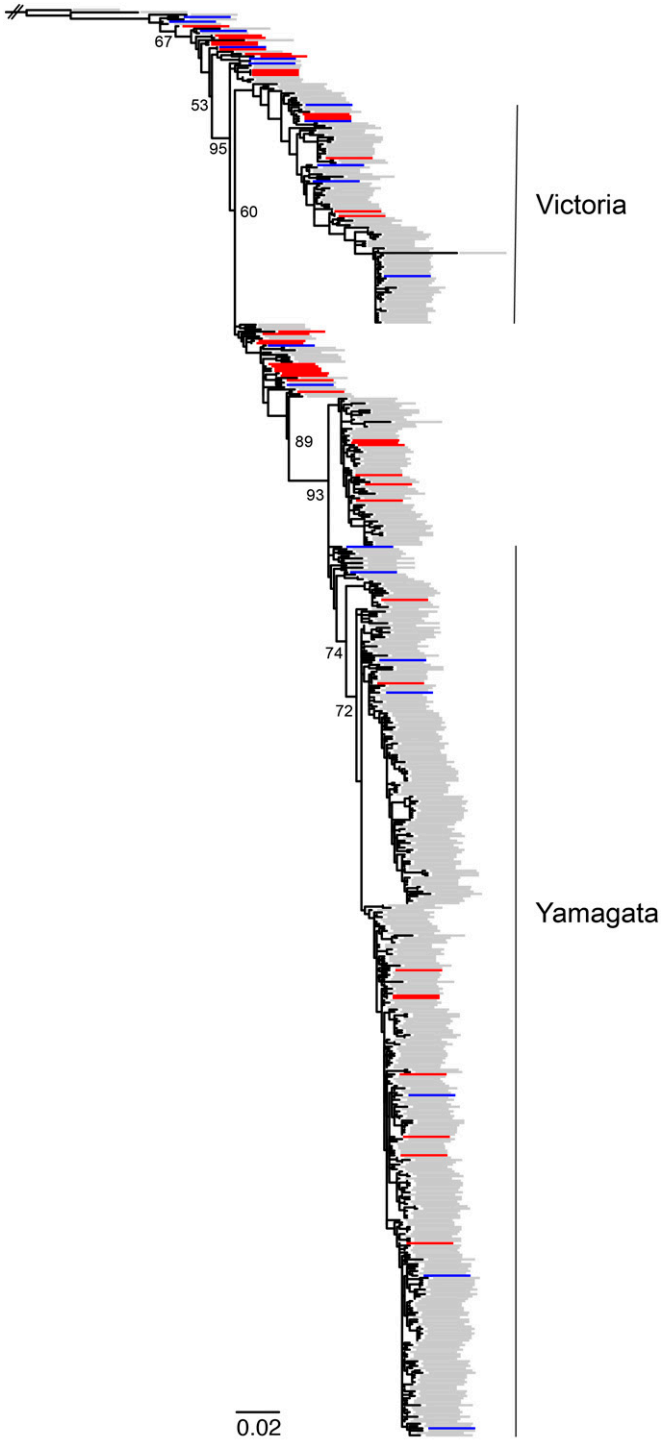

Fig. 1. Maximum likelihood phylogenetic tree inferred from 617 HA1 nucleotide sequences of viruses that span the period between 1940 and 2000. The phylogenetic tree was constructed under the GTR+G+I model. Dutch virus isolates described in this study are highlighted in red and vaccine and reference strains in blue. Bootstrap values are indicated for the main nodes in the tree. Vertical bars are shown for the B/Victoria and B/Yamagata lineages, starting from their respective reference viruses. Scale bar represents nucleotide substitutions per site.

An Antigenic Map for Historical Influenza B Viruses. To gain insight in the antigenic evolution of influenza B viruses before and shortly after the emergence of the B/Victoria and B/Yamagata lineages, an antigenic map was constructed to visualize and quantify the antigenic data. The dataset used for the antigenic map consisted of 64 antigens, including the collection of 45 Dutch cell culture virus isolates and 19 egg-passaged vaccine

strains and reference viruses from around the globe, spanning the period from 1958 to 2000 (*SI Appendix*, Table S1*A*). A panel of 21 postinfection ferret antisera raised against these viruses was selected to represent the diversity of influenza B viruses in the studied time period (*SI Appendix*, Table S2*A*). The antigenic properties of the selected viruses were analyzed in HI assays, resulting in 1,344 HI measurements (*SI Appendix*, Table S3) that were used to construct an antigenic map. First, the minimal No. of dimensions required to represent the antigenic data was determined by calculating the root mean square error (RMSE) for predicting titers when maps were produced in one to five dimensions and 10% of the HI titers were excluded randomly. Based on this analysis, the antigenic data were represented well in three dimensions (3D) (RMSE = 0.90) with no substantial improvement observed when data were analyzed in a higher No. of dimensions (RMSE 4D = 0.86, RMSE 5D = 0.87) (*SI Appendix*, Fig. S1*A*). The 3D map performed somewhat better compared with the 2D map (RMSE = 1.06). There was good correlation between the HI measurements and the distances between the respective viruses and antisera in the 3D antigenic map (R^2 = 0.86) (*SI Appendix*, Fig. S1 *B* and *C*) and the 2D antigenic map (R^2 = 0.77) (*SI Appendix*, Fig. S1*D*). Upon dimension reduction from 3D to 2D, the prominent map features were maintained (*SI Appendix*, Fig. S1 *C* and *F*), and there was a good correlation between the pairwise distances in both maps (R^2 = 0.93) (*SI Appendix*, Fig. S1*E*). The degree of uncertainty in the positioning of antigens and sera in the 2D map indicated a good coordination of all datapoints, providing confidence that the 2D antigenic map was a good representation of the HI data (*SI Appendix*, Fig. S1*F*). Based on these analyses, the 2D antigenic map was used to simplify further visualizations.

In the antigenic maps, the 64 antigens that span 42 y of evolution appeared in only three distinct clusters that we will refer to as "Early", "Victoria", and "Yamagata" to reflect the well-known reference strains for the latter clusters, B/Victoria/2/1987 and B/Yamagata/16/1988 (Fig. 2*A*). The Early antigenic cluster contained viruses that circulated from 1958 until 1974, when the first viruses emerged with antigenic properties similar to B/Victoria/2/1987. There was relatively little antigenic diversification within the Early, Victoria, and Yamagata clusters; the diameter of the Early cluster was 4.7 antigenic units (AUs), the Victoria cluster 3.7 AUs, and the Yamagata cluster 4.0 AUs, with distances of 6.8 AUs between the Early and Victoria cluster centroids, 7.9 AUs between the Victoria and Yamagata cluster centroids, and 6.8 AUs between the Early and Yamagata cluster centroids. Each antigenic cluster included multiple vaccine or reference strains, with B/England/1959, B/Singapore/3/1964, B/Roma/1967, and B/Victoria/98926/1970 for the Early antigenic cluster; B/Hong Kong/5/1972, B/Singapore/222/1979, B/USSR/100/1983, B/Ann Arbor/1/1986, B/Beijing/1/1987, and B/Shandong/7/1997 for the Victoria antigenic cluster; and B/Panama/45/1990, B/Beijing/184/1993, B/Harbin/7/1994, B/Yamanashi/166/1998, B/Sichuan/379/1999, and B/Guangdong/120/2000 for the Yamagata antigenic cluster. There was relatively little temporal signal in the positioning of antigens within each antigenic cluster, especially within the Victoria and Yamagata clusters (*SI Appendix*, Fig. S1*G*). Surprisingly, several vaccine strains were placed within 2 to 3 AUs (four to eightfold change in HI titers) in the map, as was the case for B/Singapore/222/1979, B/USSR/100/1983, B/Ann Arbor/1/86, B/Victoria/2/1987, B/Beijing/1/1987, and B/Shandong/7/1997 in the Victoria cluster and B/Harbin/7/1994, B/Yamanashi/166/1998, and B/Guangdong/120/2000 in the Yamagata cluster, indicating that these vaccine strains had rather similar antigenic properties (*SI Appendix*, Fig. S2).

Yamagata Viruses Emerged from a Victoria-like Precursor. To compare the antigenic and genetic evolution of influenza B viruses, a ML phylogenetic tree was generated using the HA1 nucleotide sequences of only the viruses used in the antigenic map (Fig. 2*B*), and the tree was color coded based on the clusters identified in the antigenic map. Viruses with the Early antigenic phenotype circulated until 1974, while viruses with Victoria-like antigenic properties emerged around 1972 and hence cocirculated for a brief period with the Early viruses (Fig. 2). Initially, the viruses with Victoria-like antigenic properties were genetically similar to the Early viruses, but they split into two lineages, one of which included B/Victoria/2/1987 itself while the second lineage diverged genetically for roughly 12 y to give rise to the lineage with B/Yamagata-like viruses around 1988. Ancestral to the B/Yamagata viruses were the well-known viruses B/Singapore/222/1979 and A/USSR/100/1983, which, despite being genetically related to B/Yamagata viruses, still had a B/Victoria-like antigenic phenotype. Altogether, these findings show that viruses with a Victoria-like antigenic phenotype emerged around 1972 and that, after the long-term coevolution

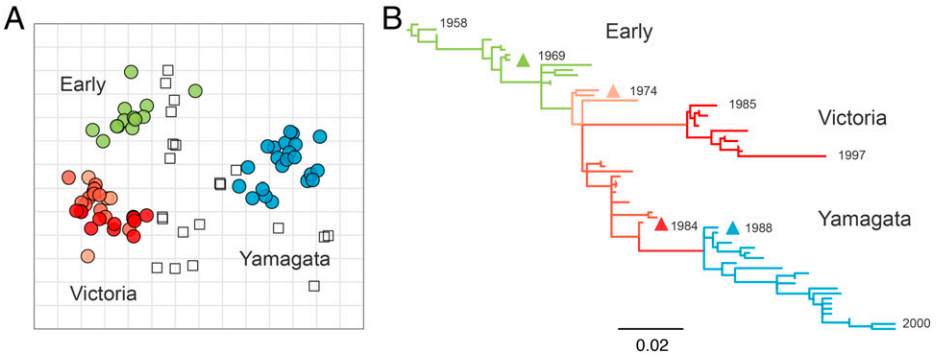

Fig. 2. Comparison of the antigenic and genetic evolution of historical influenza B viruses from 1958 to 2000. (*A*) 2D antigenic map constructed from HI titers of Dutch virus isolates and global vaccine and reference antigens and their corresponding antisera. Viruses are represented by colored circles and antisera by uncolored squares. The vertical and horizontal axis both represent antigenic distance. The spacing between the grid lines denotes 1 antigenic unit (AU) of distance, corresponding to a twofold dilution of antisera in the HI assay. Viruses are colored based on the antigenic cluster to which they belong: green for Early, blue for Yamagata, and red for Victoria. Shades of red are used to denote the different genetic lineages to which Victoria-like viruses belong. Clusters were identified by proximity of the antigens and named after the influenza B lineages they represent. (*B*) Maximum likelihood phylogenetic tree of HA1 nucleotide sequences inferred under the GTR+G+I model, color-coded based on antigenic clusters. Viruses labeled with a triangle were used as prototypes to determine the genetic basis of major antigenic change. Years of isolation are shown for several viruses for reference. Scale bar represents nucleotide substitutions per site.

of two genetic lineages of viruses with a Victoria-like phenotype, one lineage gave rise to the substantially distinct B/Yamagata antigenic variant around 1988. Antigenic change within the Early, Victoria, and Yamagata clusters was relatively limited during the 16, 25, and 12 y of study follow-up, respectively.

Substitutions Flanking the HA Receptor Binding Site (RBS) Explain Major Antigenic Change of Influenza B Viruses. To determine the amino acid (aa) substitutions in HA responsible for the distinct antigenic clusters of influenza B viruses, substitutions typical for the viruses in each cluster were identified (Fig. 3). Only two aa substitutions were observed between almost all Early and Victoria viruses: 150KN and 203TK. In contrast, as many as nine aa substitutions were observed between almost all Victoria and Yamagata viruses: 71KM, 88KR, 148NS, 149GR, 150NS, 162KR, 202VK, 203KN, and 209KN (B/Brisbane/60/2008 numbering). The effect of each of these aa substitutions on the antigenic properties of influenza B viruses was tested. To this end, the HA genes of prototype viruses B/NL/1001/1969 (NL69), B/NL/1001/1974 (NL74), B/NL/312/1984 (NL84), and B/Yamagata/16/1988 (Yam88) were cloned and recombinant viruses were rescued to investigate the cluster transitions from Early to Victoria (NL69, NL74) and from Victoria to Yamagata (NL84, Yam88) (Fig. 4). A substitutions were introduced into the HA of prototype viruses to generate mutant viruses for testing in HI assays against the antisera used to construct the antigenic map.

Substitution 150KN in the Early virus NL69 resulted in a marked decrease of titers to Early antisera, while substitution 203TK in NL69 resulted in a marked increase of titers to Victoria antisera. When both substitutions were combined, NL69 reacted like a typical Victoria-like virus (Fig. 4*A* and *SI Appendix*, Fig. S3*A*). The impact of the reverse substitutions on the Victoria-like virus NL74 were less clear, possibly as a result of overall low reactivity of the NL74 recombinant virus in the HI assays. Nevertheless, the combination of 150NK and 203KT in NL74 HA had a large impact on antigenic properties, with reduced titers against Victoria antisera and increased titers to Early antisera, bringing the NL74 mutant virus in close proximity to the Early cluster in the antigenic map (Fig. 4*B* and *SI Appendix*, Fig. S3*B*). The incomplete phenotype change of the NL74 double-mutant virus may be attributable to (unknown) epistatic interactions, given that no other aa substitutions were associated consistently with the Early and Victoria antigenic clusters (Fig. 3).

To investigate the molecular basis of the antigenic differences between Victoria and Yamagata viruses, the impact of single aa

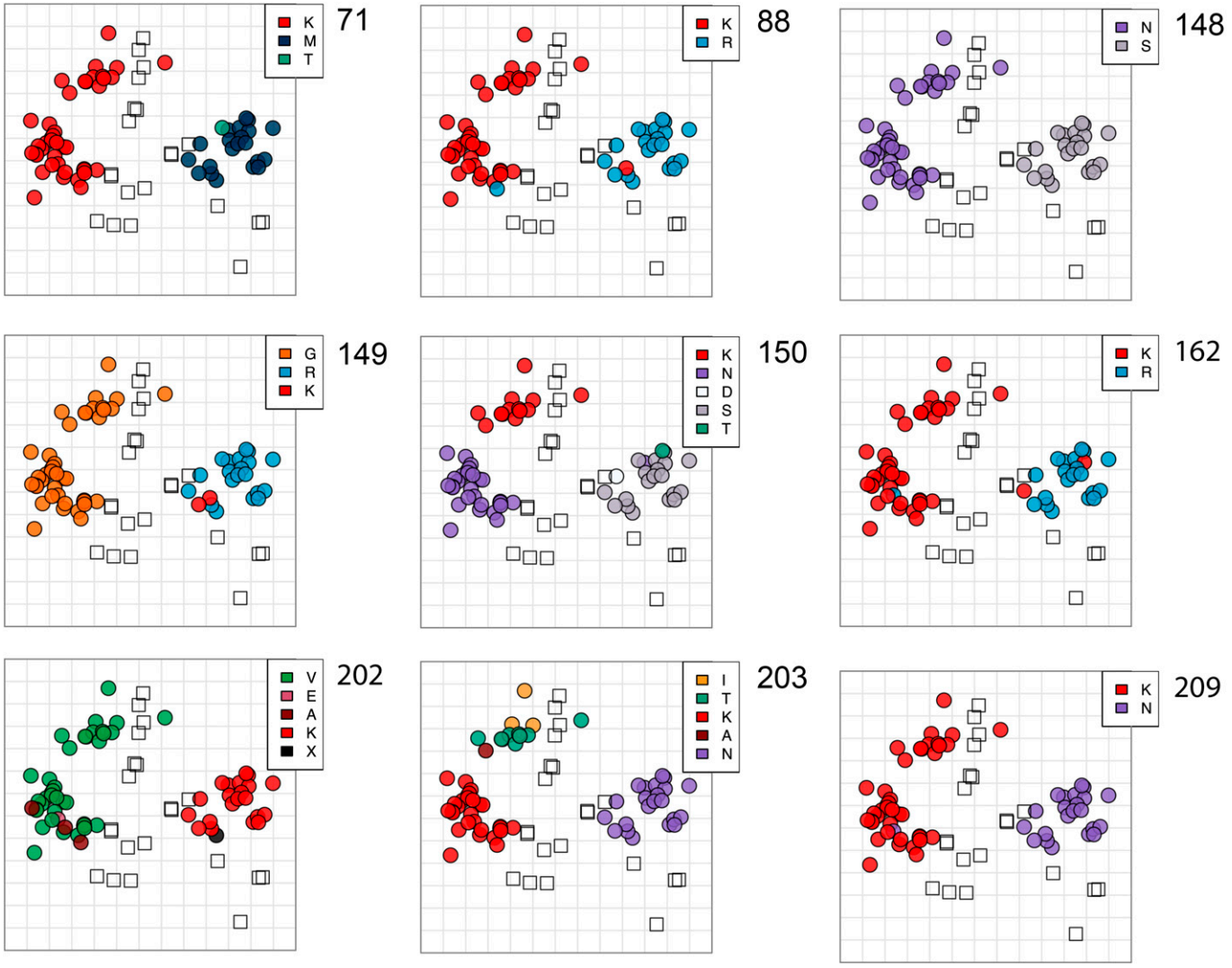

Fig. 3. Fixation of amino acid substitutions across antigenic cluster boundaries. Antigenic map is the same as shown in Fig. 2 but color coded based on amino acid residues at positions 71, 88, 148, 149, 150, 162, 202, 203, and 209. Numbering is according to B/Brisbane/60/2008 HA.

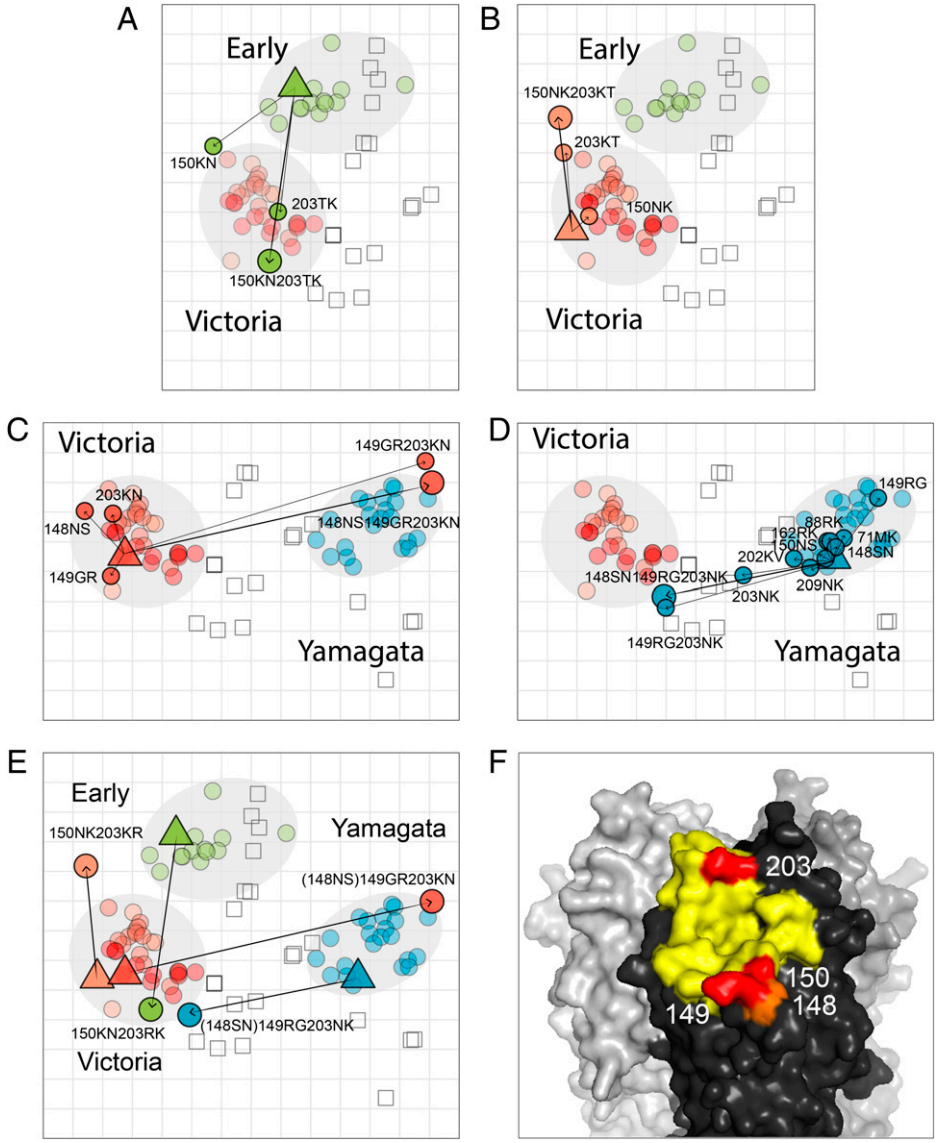

Fig. 4. Antigenic effect of amino acid substitutions in HA on cluster transitions. Antigenic maps show the effect of individual or combined amino acid substitutions on recombinant prototype viruses. Wild-type viruses are shown as colored triangles, mutant viruses as colored circles, and antisera as uncolored squares. Arrows indicate the antigenic distance and direction caused by amino acid substitutions. Viruses are colored based on antigenic clusters, and cluster boundaries are schematically represented by gray-shaded ovals. The vertical and horizontal axis both represent antigenic distance. The spacing between the grid lines denotes 1 AU of distance, corresponding to a twofold dilution of antisera in the HI assay. (*A* and *B*) Analysis of the Early to Victoria cluster transition, with substitutions introduced (*A*) in the Early prototype virus NL69 and (*B*) in the Victoria prototype virus NL74. (*C* and *D*) Analysis of the Victoria to Yamagata cluster transition, with substitutions introduced (*C*) in the Victoria prototype virus NL84 and (*D*) in the Yamagata prototype virus Yam88. (*E*) Overview of amino acid substitutions explaining the cluster transitions. (*F*) Crystal structure of B/Yamanashi/166/1998 HA (PDB 4M40) with key positions explaining cluster transitions shown in red and position 148 in orange to mark its more-modest effect and the RBS in yellow. Numbering is according to B/Brisbane/60/2008 HA.

substitutions was first tested in the Yam88 HA backbone. Most single aa substitutions (71MK, 88RK, 148SN, 150NS, 162RK, 202KV, and 209NK) had very limited impact on the antigenic properties of Yam88, primarily resulting in moderately reduced titers to Yamagata antisera. In contrast, single aa substitutions 149RG and 203NK had much larger impact on antigenic properties, through a substantial reduction of titers to Yamagata antisera and a substantial increase in titers to Victoria antisera, respectively (Fig. 4*D* and *SI Appendix*, Fig. S4*B*). A Yam88 mutant containing both 149RG and 203NK resembled Victoria-like viruses in reactivity to the Victoria and Yamagata antisera and was positioned close to the Victoria cluster in the antigenic map. Likewise, the introduction of single aa substitutions 149GR and 203KN in HA of NL84 had only a modest impact on antigenic properties of this Victoria-like virus, but the combination of the two aa substitutions resulted in substantially reduced reactivity to Victoria antisera and increased reactivity to several Yamagata antisera. Consequently, the double-mutant virus was positioned close to the Yamagata cluster in the antigenic map (Fig. 4*C* and *SI Appendix*, Fig. S4*A*). No other aa substitutions were identified to contribute further to the antigenic phenotype of Victoria and Yamagata viruses, except perhaps 148NS. Substitution 148NS in NL84 resulted in reduced titers to Victoria antisera and, when combined with 149GR/203KN, resulted in a slight further increase of titers to the Yamagata antisera. Although 148SN alone had only a modest impact in the Yam88 backbone, it resulted in a further increase of titers to the Yamagata antisera when combined with 149RG/203NK (*SI Appendix*, Fig. S4). Therefore, we concluded that 149GR and 203KN were the main determinants of antigenic differences

between the Victoria-like and Yamagata-like viruses, with potentially some additional effect of 148NS.

To confirm that the effect of the identified aa substitutions on antigenic properties was not due to HI artifacts, recombinant viruses with and without the aa substitutions at positions 148, 149, 150, and 203 in HA were also tested in a virus neutralization (VN) assay. There was correspondence of antibody titers as determined by VN and HI assays ($R^2 = 0.56$), but the HI assay was more sensitive and thus low antibody titers in the HI assay were missed in the VN assay (*SI Appendix*, Fig. S5).

The aa substitutions at positions 148–150 and 203 that largely explained the major antigenic changes between Early and Victoria-like viruses and between the Victoria-like and Yamagata-like viruses (Fig. 4*E*) were colored in the crystal structure of influenza B virus HA. This illustrated that each of these aa substitutions was located directly flanking the RBS (Fig. 4*F*), where they might contribute to the escape from binding by antibodies that would directly block HA-receptor interactions.

No Direct Impact of Indels on Major Antigenic Change of Influenza B Virus. Since indels in the HA gene—in particular around aa positions 163–165—have shaped the evolution of influenza B viruses over time (12), we assessed evidence for the impact of indels on the antigenic properties of influenza B viruses. Early viruses showed wide variation at positions 163–165, having zero, one, two, or three aa deletions relative to B/Brisbane/60/2008, while displaying high similarity in antigenic properties (Fig. 5). Likewise, B/Victoria-like and B/Yamagata-like viruses showed substantial variation in indels as shown in the ML phylogenetic tree (Fig. 5*A*), with Victoria-like viruses having zero, two, or three aa deletions and Yamagata-like viruses having one or two aa deletions, but this did not necessarily result in major antigenic changes in the antigenic map (Fig. 5*B*) or the original HI data (*SI Appendix*, Table S4). To confirm whether indels had an impact on the effect of aa substitutions associated with antigenic change, two viruses were chosen from the Early cluster that had zero deletions (NL69) or three deletions (B/NL/1000/1971, NL71) and aa substitutions 150KN and 203TK were introduced, alone and in combination. Irrespective of the HA background used, the aa substitutions had a comparable impact on the antigenic properties of the original viruses (Fig. 5*C* and *SI Appendix*, Fig. S6), indicating that, although indels may have shaped the evolution of influenza B virus, they did not have a direct impact on antigenic properties.

Recent Emergence of B/Victoria Variants with aa Substitutions at Positions 150 and 203. Starting from 2019, new variants of the B/Victoria lineage with substitutions at positions 150 and 203 in HA were sampled across multiple continents (https://GISAID.org). As of 2021, the majority of the sampled viruses had 150K with or without 203R, while the older Victoria-like viruses had 150N and 203K. The 150K substitution could be considered a reversion, given that all Early viruses that circulated until 1974 had 150K but with 203T/I/A. To assess whether the antigenic properties of recent variants changed as a result of the substitutions at these two positions, we selected representative Dutch cell culture viruses circulating between 2000 and 2020 and vaccine strains from the same time period (*SI Appendix*, Table S1*B*) and tested them in HI assays against a panel of five antisera raised against historical Early and Victoria viruses and recent viruses of the B/Victoria lineage (*SI Appendix*, Table S2*B*). Interestingly, the 2019–2021 B/Victoria lineage viruses with 150K had high titers with antisera against Early viruses that circulated in the 1960s and 1970s, while Victoria viruses with 150N showed no or very low titers (Table 1). Similarly, Early viruses from 1967 or 1970 revealed high titers with antisera raised against viruses with 150K from 2019 and 2021 but had no or low reactivity with antisera raised against Victoria viruses with 150N. While recent viruses with 150K/203K were still recognized well by antisera raised against viruses with 150N/203K, titers to viruses with 150K/203R dropped considerably, thus also demonstrating an impact of 203KR on antigenic properties (Table 1). These data indicate that recent B/Victoria viruses have acquired aa substitutions in HA that substantially reversed the antigenic phenotype toward Early viruses that circulated prior to 1974.

Discussion

Analyses of the antigenic properties of a unique collection of historical cell-passaged influenza B viruses together with molecular analyses provided insights into the early events that led to the emergence of the B/Victoria and B/Yamagata lineages. Remarkably, an influenza B virus antigenic map spanning a 42-y period revealed only three major antigenic clusters of Early, Victoria-like, and Yamagata-like viruses, within an antigenic space of ∼11 AUs (i.e., 11 $\log_2$ dilutions of antisera). This is in sharp contrast to the ∼45 AUs covered by 11 antigenic clusters in 35 y of antigenic evolution of A/H3N2 virus (29), confirming the relatively slow rate of antigenic drift of influenza

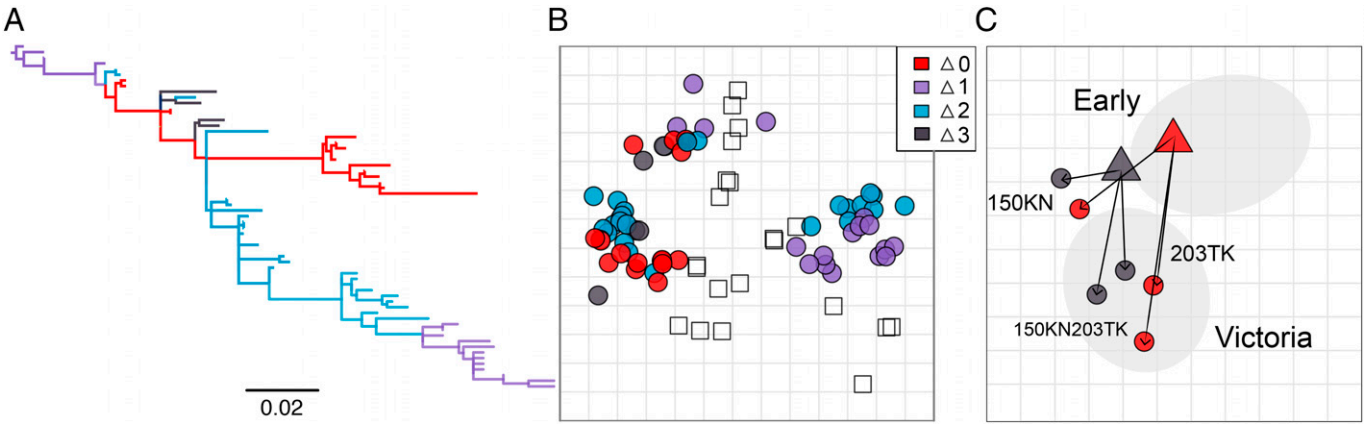

Fig. 5. Effect of indels on the antigenic properties of historical influenza B viruses. (*A*) The maximum likelihood phylogenetic tree of Fig. 2 was colored based on the presence of zero (red), one (purple), two (blue), or three (gray) deletions in the 160 loop of the HA protein. (*B*) The antigenic map of Fig. 2 was color coded based on the presence of deletions in the 160 loop of the HA protein, matching *A*. (*C*) Antigenic map displaying the effects of aa substitutions 150KN and 203TK alone and in combination in NL69 (red triangle) and NL71 (gray triangle) viruses containing zero and three deletions in the 160 loop, respectively.

Table 1. Effect of aa substitutions at positions 150 and 203 in recent B/Victoria lineage viruses

Virus	Deletion 160-loop	Aa* 150|203	Vic/70 KT	Beij/87 NK	Wash/19 NK	Cro/19 KK	Aus/21 KR
Rome/67	0	KT	480	<10	<10	640	480
Vic/70	2	KT	**640**	10	10	640	640
Vic/87	0	NK	<10	640	240	160	<10
Beij/87	0	NK	<10	**640**	240	120	<10
Col/17	2	NK	<10	480	1280	240	30
Wash/19	3	NK	30	320	**1920**	320	80
IvC/20	3	KK	320	80	1280	1280	960
Cro/19	3	KK	320	640	1280	**1280**	640
Par/20	3	KR	320	80	320	640	1280
Aus/21	3	KR	320	40	160	640	**1280**

*Reactivity of viruses with 150K/203T (KT), 150N/203K (NK), 150K/203K (KK), or 150K/203R (KR) was tested with antisera in the HI assay. Bolded and underlined text represents homologous HI titers.

B virus. While the B/Victoria genetic lineage is thought to have its origins in China around 1975 (5), viruses with Victoria-like antigenic properties already circulated both within and outside of Asia at an earlier time point, as illustrated by Victoria-like strains B/Hong Kong/5/1972 and B/NL/1000/1974 in this study. These viruses could easily be misclassified if only their genetic characteristics were taken into account, since they are genetically similar to early viruses circulating prior to the emergence of the B/Victoria-like viruses. Interestingly, in 1973, the World Health Organization took notice of the low reactivity between B/Hong Kong/5/1972 and antisera raised to viruses from 1967 until 1971 and advised to incorporate a B/Hong Kong/5/1972-like antigen in addition to the previous vaccine strain B/Victoria/98926/1970 in a trivalent vaccine along with the A/H3N2 component (30). In the present study, B/Victoria/98926/1970 and B/Hong Kong/5/1972 are indeed placed in different clusters in the antigenic map.

Viruses with a Victoria-like phenotype continued to diverge for roughly 12 y until a new lineage of viruses with distinct antigenic properties emerged, named after B/Yamagata/16/1988. The emergence of these Yamagata-like viruses thus resulted upon continuous coevolution of genetic variants with a Victoria-like phenotype and illustrates the importance of monitoring cocirculating genetic lineages of influenza viruses for timely identification of antigenic drift. Cocirculation of multiple lineages of influenza A/H3N2 and A/H1N1$_{pdm09}$ viruses has also been noted in recent years, due to increased sequencing efforts or increased cocirculation of genetic variants (31).

Influenza B virus antigenic data revealed two major antigenic changes between 1958 and 2000, and the aa substitutions responsible for these changes in phenotype were mapped to three or four positions in two sites directly flanking the RBS. Additional minor antigenic changes were also observed within each cluster, but these appeared as relatively small temporal variations that were not addressed further in this study. Of note, antigenic changes have also been noted after this study period (20, 32, 33). Both position 203 and the region 148–149–150 in HA appear to be important for the antigenic phenotype of influenza B viruses since changes here occurred in both cluster transitions. Early studies identified substitutions at these positions to affect binding and inhibition by mAbs (23, 24, 34). All four positions were also found to be under strong positive selection in genetic analyses (35). These aa changes responsible for the antigenic differences between Early, Victoria, and Yamagata viruses involved large changes in the biophysical properties of the aas affecting charge and volume, as was shown for A/H3N2 viruses (32). Interestingly, positions 203 and 150 are equivalent to positions 193 and 145 in the H3N2 virus, respectively, which were also identified as major antigenic determinants (32). Also similar to A/H3N2 viruses, as well as A/H1N1pdm, A/H2N2, and A/H5N1 viruses, was the fact that major antigenic changes were primarily due to aa substitutions that occurred immediately adjacent to the RBS, where they may contribute most effectively to evasion from antibodies that directly neutralize HA-receptor interactions (32, 36, 37). An interesting observation for the influenza B viruses was the apparent immunodominance of these sites as measured with ferret antisera. Sera raised against Early viruses reacted well with viruses with 150K but less so with 150N. Sera raised against the Victoria-like viruses reacted well with 203K but less so with 203T or 203N. Sera raised against the Yamagata-like viruses reacted well with viruses with 149R (and 148S) but less so with 149G (and 148N) (*SI Appendix*, Figs. S3 and S4). Thus, single substitutions at a critical site in HA can have substantial impact on the antibody responses upon infection, relatively independent of the other antigenic site(s).

The 190 helix in the HA structure—which includes position 203—was shown to have a dominant role in eliciting HI antibodies in mice and ferrets, followed by the 160 and the 150 loops, the latter of which includes positions 148, 149, and 150 (38). In humans, HI antibody responses to the 190 helix and 150 loop were also shown to dominate in adults, although responses to noncanonical antigenic sites were suggested to become increasingly important in older populations (38). While the 120 and 160 loops and noncanonical antigenic sites were not involved in the major antigenic changes between the Early, Victoria, and Yamagata clusters, it cannot be excluded that they played a role in the smaller antigenic differences observed within the different antigenic clusters that were not mapped in the present study. It is also important to note that our study focused on HI/VN antibodies and not on antibodies with other effector functions (e.g., via interactions with Fc receptors) or antibodies to other viral proteins that may drive the antigenic evolution of influenza viruses and contribute to protection (39, 40).

While indels in the 160 loop have shaped the genetic evolution of influenza B virus (12, 13), their impact on antigenic properties has been less clear. In this study, indels in the 160 loop did not have direct effects on the antigenic properties of the influenza B viruses during the studied time period. However, it cannot be excluded that indels alone or in combination with other substitutions may play a role in more-recent strains. The recent acquisition of deletions in the B/Victoria lineage, after remaining constant for 4 decades, and the emergence of substitutions at positions 150 and 203 concurrently may

suggest a possible association between indels and aa substitutions with impact on antigenic properties. Although such association between indels and substitutions with antigenic impact was not observed for the Early to Victoria change, epistatic interactions could be at play in more-recent virus lineages. While the antibody responses to the major antigenic sites of influenza B were shown to be comparable in mice, ferrets, and humans (38), it is possible that the 160 loop displays different immunogenicity in ferrets as compared with humans.

Given the rather narrow antigenic space covered within each cluster (<4.7 AUs) and the lack of clear temporal patterns, it was surprising to find six vaccine strains in the Victoria cluster and five in the Yamagata cluster. Some of these vaccine strains within the Victoria and Yamagata clusters indeed showed relatively high antigenic similarity, although fourfold differences in two-way antibody titrations were seen (*SI Appendix*, Fig. S2). It is likely that temporal and geographical patterns of the dominating lineages between 1972 and 2000 and the problems associated with egg adaptation of vaccine strains have contributed to more frequent vaccine updates for influenza B virus than strictly required based on their antigenic evolution alone. Egg-passaged viruses may display higher HI titers than viruses exclusively passaged in mammalian cells, and antigenic properties may change due to, e.g., alterations of the RBS and glycosylation sites (41). It has been shown previously that influenza B viruses that belong to different genetic clades may have antigenic properties that are very similar (16). Avoidance of egg-adaption mutations remains important in influenza virus surveillance and the production of influenza seed strains and vaccines.

Viruses with substitutions at position 150 alone or in combination with 203 have recently (>2019) emerged within the B/Victoria lineage and have since become the dominant circulating influenza B viruses. In HI assays, these recent viruses indeed antigenically resembled viruses circulating prior to the emergence of the B/Victoria and B/Yamagata lineages, illustrating that recent B/Victoria-like isolates have undergone a phenotypic "reversion". Although the aa present at position 203 in recent isolates was not the same as that in early strains, the K203R substitution had an impact on the antigenic properties of the virus. Interestingly, while viruses harboring only the K substitution at position 150 were well neutralized by both the Early and typical Victoria antisera, viruses with both substitutions at 150 and 203 were still neutralized by Early but to a lesser degree by the typical Victoria antisera. The seed strain recommended in seasonal vaccines for the 2022 season in the Southern Hemisphere and 2022–2023 season in the Northern Hemisphere was B/Austria/1359417/2021, harboring substitutions at both 150 and 203 positions (42).

The present study sheds light on the molecular mechanisms driving the emergence and antigenic characteristics of the Victoria-like and Yamagata-like influenza B viruses. Retrospective investigation of such events can help better understand how novel antigenic variants emerge in order to improve vaccine-strain selection and pandemic preparedness.

Materials and Methods

Cells. Cells were grown essentially as described in ref. (32). 293T cells were cultured in Dulbecco's modified Eagle's medium (Lonza, Breda, The Netherlands) supplemented with 10% fetal bovine serum (Greiner), 1% nonessential aas (NEAAs) (Lonza), 1 mM sodium pyruvate (Gibco), 2 mM glutamine (L-glu, Lonza), 100 IU/mL penicillin (PEN) (Lonza), and 100 μg/mL streptomycin (STR) (Lonza). Madin–Darby canine kidney (MDCK) cells were cultured in Eagle's minimal essential medium (Lonza) supplemented with 10% fetal calf serum, 1% NEAAs, 1.5 mg/mL sodium bicarbonate (Lonza), 10 mM Hepes (Lonza), 2 mM glutamine, 100 IU/mL PEN, and 100 μg/mL STR.

Viruses. A collection of 64 influenza B viruses spanning the period from 1958 until 2000 was used for the phylogenetic and antigenic analysis. Of those, 45 viruses were isolates collected in the Netherlands and exclusively passaged in mammalian cells to preserve the genetic and antigenic properties of the original isolates and 19 were vaccine or reference strains. The passage history of all viruses used in this study is provided in *SI Appendix*, Table S1. The HA sequences were determined for the most-recent virus passage by Sanger sequencing and are available from the GISAID EpiFlu database (www.gisaid.org), with accession Nos. provided in *SI Appendix*, Table S1. A high-yield unidirectional reverse genetics system for influenza B/Yamagata/1/1973 and pHH21 plasmids containing the HA and neuraminidase (NA) genes from B/Yamagata/16/1988 were a kind gift from Prof. Yoshihiro Kawaoka, Influenza Research Institute, University of Wisconsin–Madison (43). HA genes from B/Netherlands/1001/1969, B/Netherlands/1000/1971, B/Netherlands/1001/1974, and B/Netherlands/312/1984 were cloned into pHW2000 reverse genetics plasmids and, together with pHH21 plasmids encoding the B/Yamagata/16/1988 HA and NA genes, were used to generate recombinant high-yield viruses. A substitutions were introduced into the plasmids encoding the different HA genes to generate mutant viruses. Introduction of mutations was done with specific primers and Phusion High-Fidelity DNA Polymerase (Thermo Scientific). The presence of correct mutations and absence of undesired mutations was confirmed by Sanger sequencing of the HA gene. All isolates and recombinant viruses were propagated at 33 °C.

ML Phylogenetic Analysis. All available influenza B HA1 sequences with a minimum length of 1,000 nucleotides and a collection date prior to the 31st of December 2000 were retrieved from GISAID (44) (gisaid.org) on April 28, 2021. After duplicate entries were removed, the downloaded sequences were combined with the 64 HA1 nucleotide sequences generated for this study, resulting in a final dataset containing 617 viruses sampled worldwide. Nucleotide sequences were aligned with MAFFT version 7, and indels were inspected manually. A ML phylogenetic tree was inferred with PhyML version 3.3 under a general-time reversible substitution model with a proportion of invariant sites and a gamma distribution of rate variation, all estimated from the data by JModeltest 2.1 (https://github.com/ddarriba/jmodeltest2). A ML phylogenetic tree containing the 64 HA1 nucleotide sequences from this study was obtained in a similar way. Bootstrap resampling analysis was performed with 100 replicates. The trees were visualized, rooted, and colored using FigTree version 1.4.4 (http://tree.bio.ed.ac.uk/software/figtree/).

Sera and Serological Assays. Ferret studies were carried out in strict compliance with the Dutch legislation for the protection of animals used for scientific purposes (2014, implementing EU Directive 2010/63). Research was conducted under a project license from the Dutch competent authority (license No. AVD101002015340), and the study protocol was approved by the institutional Animal Welfare Body (Erasmus MC permit No. 15–340-21). Animal welfare was monitored on a daily basis. Postinfection sera were generated in male ferrets upon intranasal inoculation with 1 mL virus stock, and serum was collected after 14 d. All sera were treated overnight at 37 °C with Burnet's receptor-destroying enzyme (filtered from cultures of *Vibrio cholerae*) at a 1:6 ratio and inactivated at 56 °C for 1 h (45). An overview of the sera used for the study can be found in *SI Appendix*, Table S2. The treated sera were used in HI assays essentially as shown before (46). Briefly, twofold serial dilution of the sera in PBS, starting at a 1:20 dilution, was mixed with 25 μL of virus containing four hemagglutinating units and was incubated at 37 °C for 30 min. Upon incubation, 25 μL of 1% turkey red blood cells (TRBCs) was added to the mixture and incubated for 1 h at 4 °C. The plates were then read, and the HI titer was registered as the reciprocal value of the highest dilution of serum that completely inhibited agglutination of TRBC. VN assays were performed as described previously (47). Briefly, antisera were heated for 30 min at 56 °C and twofold serial dilutions of the antisera starting at 1:20 were mixed 1:1 with 100 tissue culture infectious dose$_{50}$ of virus. After incubation at 37 °C for 2 h, virus–antisera mixtures were transferred to 96-well plates containing confluent MDCK cells that were washed twice with PBS prior to inoculation. After incubation at 37 °C for 2 h, inoculum was replaced with 200 μL infection medium and cells were incubated for 3 d at 37 °C. Endpoint dilutions were read by hemagglutination assay.

Antigenic Cartography. Antigenic maps were built to facilitate quantification and visualization of antigenic data obtained from HI assays as described previously (29). The construction of antigenic maps was facilitated by the Racmacs R package version 1.1.18 (https://acorg.github.io/Racmacs/). In an antigenic map, the distance between antiserum point S and antigen point A corresponds to the difference between the $\log_2$ of the maximum titer observed for antiserum S against any antigen and the $\log_2$ of the titer for antiserum S against antigen A. Through modified multidimensional scaling methods, antigens and antisera are placed in an antigenic map to best satisfy the distances specified by the HI titer. The distance between points represents antigenic distance as measured by the HI assay, in which the distances between antigens and antisera are inversely related to the $\log_2$ HI titer. Since antisera and antigens are tested against numerous other antigens and antisera, multiple measurements can be used to determine the position of the antigen and antiserum points in an antigenic map, allowing for maps to be interpreted to higher resolution. Antigenic maps were constructed using 1,000 optimizations, with no minimum column basis set.

Data, Materials, and Software Availability. Sequences generated for this study have been deposited in GISAID. (The passage history of all viruses used in this study is provided in *SI Appendix*, Table S1. The HA sequences were determined for the most recent virus passage by Sanger sequencing and are available from the GISAID EpiFlu database (www.gisaid.org) (48), with accession Nos. provided in *SI Appendix*, Table S1.). All genome sequences and associated metadata used in this study are published in GISAID. Details about the contributors of each individual sequence with details such as accession number, virus name, collection date, originating lab and submitting lab and the list of authors can be found in *SI Appendix*, Table S5.

All other data are included in the manuscript and/or *SI Appendix*.

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