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Article

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Genetic characterization of influenza A and B viruses circulating from 2017 to 2023 In Guizhou Province,China

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Abstract

Purpose This study analyzed the genetic characteristics of influenza viruses circulating in Guizhou Province from 2017 to 2023 and their match with seasonal vaccine strains.

Methods Based on hemagglutinin (HA) gene sequences, we performed phylogenetic analysis and assessed amino acid homology, antigenic site, and glycosylation site variations in A(H1N1), A(H3N2), B Victoria (BV), and B Yamagata (BY) viruses.

Results HA homology with vaccine strains was highly conserved for H1N1, BV, and BY (median >98.7%). In contrast, H3N2 viruses showed significantly lower homology (median 98.0%; IQR 97.81% – 98.00%) and broader variation (96.55% – 99.09%). Phylogenetically, all H1N1 strains clustered within 6B.1A, H3N2 within 3C.2a1b, BV within 1A.3a.1, and BY within Y3. Notably, some H3N2 strains with low homology formed an independent cluster and commonly carried the S193F substitution in antigenic site B, suggesting potential antigenic drift. Key mutations in antigenic and glycosylation sites were identified across all subtypes, particularly in H3N2 and BV.

Conclusion Despite overall high homology, the observed genetic variability—especially the emergence of a distinct H3N2 cluster with antigenic site mutations—highlights the continuous evolutionary potential of influenza viruses in Guizhou. These findings underscore the need for enhanced genomic surveillance to monitor antigenic drift, inform the timely reformulation of annual vaccine compositions, and support evidence-based recommendations on seasonal influenza vaccination.

Key words: Influenza virus; Hemagglutinin; Genetic characterization; Vaccine strain matching; Antigenic drift; Molecular surveillance

1.Introduction

Influenza is a highly contagious respiratory illness caused by RNA viruses capable of infecting humans. Annually, influenza affects approximately 5–15% of the global population, resulting in an estimated 250,000 to 500,000 deaths worldwide[1, 2], thereby posing a serious threat to public health.

Influenza viruses belong to the family Orthomyxoviridae and are classified into four types—A, B, C, and D—based on antigenic and genetic differences in their nucleoprotein and matrix protein[3]. Influenza A viruses exhibit high antigenic variability, strong replication capacity, and the ability to undergo genetic reassortment, making them the primary cause of influenza pandemics[3]. Although often overlooked, influenza B viruses also contribute significantly to seasonal outbreaks, particularly among children, and can dominate in certain seasons[4-6]. In China, the main circulating strains responsible for seasonal influenza include A(H1N1), A(H3N2), B Victoria（BV）, and B Yamagata（BY）[7], which often alternate in prevalence across years[8].

Influenza viruses evolve rapidly, accumulating mutations that enable them to evade both natural and vaccine-induced immunity. Much of this evolution occurs in the hemagglutinin (HA) surface glycoprotein[9], which serves as the primary target of neutralizing antibodies and a key component of influenza vaccines. Although annual vaccination remains the most effective strategy to reduce influenza-related morbidity and mortality, its effectiveness can be limited when antigenic mismatches occur between vaccine and circulating strains[10, 11]. The HA protein contains antigenic sites and receptor-binding domains; mutations in these regions may alter antigenicity, receptor specificity, and virulence[12]. Continuous accumulation of mutations in antigenic sites drives viral evolution and the emergence of new strains[13]. The frequent appearance of new genetic variants capable of replacing previously circulating strains underscores the need for regular updates to vaccine formulations.

Amino acid substitutions in HA may also lead to changes in glycosylation patterns, which can facilitate immune evasion by shielding epitopes, stabilizing HA structure, or modulating receptor-binding affinity[14-16]. Therefore, continuous virologic surveillance is essential to detect emerging strains with potential for enhanced transmissibility, virulence, or resistance to antivirals.

To better prevent and control seasonal influenza, based on the comparison with existing vaccine strains, this study analyzed the genetic and molecular characteristics of influenza A and B viruses

detected in Guizhou from 2017 to 2023.

2. Materials and Methods

2.1 Cell culture and virus infection

2.1.1. Cell Culture

Madin-Darby canine kidney (MDCK) cells (obtained from the National Influenza Center) were cultured in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 25 mM HEPESr, and 1% penicillin-streptomycin at 35°C in a humidified incubator with 5% CO_2 for 2-3 days.

2.1.2. Virus Inoculation and Propagation

For virus isolation, MDCK cells were seeded in T-25 flasks and grown to approximately 90% confluence. Prior to infection, the cell monolayer was washed three times with phosphate-buffered saline (PBS) to remove residual serum. Next, 1 mL of clinical specimens, which had been identified as influenza A or B virus-positive by RT-PCR, was inoculated onto the cells. The cultures were incubated for 1 hour at 35°C to allow for virus adsorption, with gentle rocking every 15 minutes. Following the adsorption period, the inoculum was aspirated, the cells were washed once with PBS, and then 5 mL of the serum-free DMEM (used as the virus growth medium) containing 2 μg/mL TPCK-trypsin (Tosylamide Phenylethyl Chloromethyl Ketone-treated trypsin; Sigma-Aldrich, Type VIII from bovine pancreas) was added to support viral propagation.

2.1.3. Harvest of Viral Supernatant

The infected cell cultures were incubated at 35°C and monitored daily for the development of cytopathic effect (CPE, e.g., cell rounding and detachment). Once significant CPE was observed (typically 3–7 days post-infection), the culture supernatant was harvested by centrifugation at 3,000 ×g for 10 minutes at 4°C. The clarified supernatant was then aliquoted and stored at -80°C for further viral titration and characterization.

2.1.4. Hemagglutination and hemagglutination inhibition assays

The hemagglutination (HA) activity of the harvested culture supernatant was tested using guinea pig or human type O erythrocytes. Samples negative for HA were blindly passaged once, Samples with low HA titers (<1:8) were passaged at dilutions ranging from 10^{-1} to 10^{-3} to amplify the virus. Viral type and subtype were subsequently confirmed by hemagglutination inhibition (HI) assays using reference antisera provided by the National Influenza Center.

2.2 Sequencing and Data Collection

A total of 312 real-time RT-PCR positive clinical specimens from influenza-like illness (ILI) cases, collected between January 2017 and December 2023, were selected for genetic analysis. The specimens comprised influenza A(H1N1) (n=31), A(H3N2) (n=86), BV(n=171), and BY (n=24). The annual distribution of strains is detailed in Table 1.

Viral RNA was extracted from these specimens using a Viral DNA/RNA Extraction Kit (Magnetic Bead Method; Xi'an Tianlong Science and Technology Co., Ltd.). Subsequently, the HA gene was amplified using subtype-specific primers with the PrimeScript One Step RT-PCR Kit Ver.2 (Dye Plus; TaKaRa). The resulting amplicons were purified and sequenced via the Sanger method at either the National Influenza Center or Sangon Biotech (Shanghai). For phylogenetic and comparative analysis, reference sequences representing known clades and the WHO-recommended Northern Hemisphere vaccine strains for the 2017–2023 seasons were downloaded from the GISAID database.

Table 1 Number of different subtype/lingage HA nucleotide sequences were used in this study in Guizhou from 2017 to 2023.

Subtype/Lingage	No of 2017 strains	No of 2018 strains	No of 2019 strains	No of 2020 strains	No of 2021 strains	No of 2022 strains	No of 2023 strains
H1N1	8	3	12	0	0	0	8
H3N2	8	2	14	0	0	57	5
B/Victoria	5	4	21	34	91	16	0
B/Yamagata	9	8	7	0	0	0	0

2.3 Amino acid homology analysis

The "DISTANCE" tool in MEGA 12.0.10 software was used to calculate the amino acid sequence homology of HA protein between each epidemic strain and its corresponding vaccine strain, and the homology percentage was recorded. For each influenza virus subtype, descriptive statistics were calculated to characterize the distribution of homology values throughout the study period (2017-2023). This includes the median used to represent the concentration trend, the first and third quartiles used to define the interquartile range, and the minimum and maximum values used to define the total range. The interquartile range contains the middle 50% of the data and is used as the main indicator to measure the degree of dispersion.

2.4 Phylogenetic Analysis

Phylogenetic analysis was performed using MEGA software (version 12.0.10). Maximum likelihood trees were constructed with the best-fit nucleotide substitution models, which were the Hasegawa-Kishino-Yano model with gamma distribution (HKY+G) for H1N1 and influenza B viruses, and HKY+G with invariant sites (HKY+G+I) for H3N2 viruses. The statistical confidence of the tree topology was assessed with 1000 bootstrap replicates. The final phylogenetic trees were visualized and annotated using the online tool Chiplot (https://www.chiplot.online/).

2.5 Amino Acid Mutation Analysis

Amino acid sequences were translated from nucleotide sequences using the standard genetic code in MEGA software. Multiple sequence alignment was then performed using the ClustalW algorithm to identify amino acid substitutions.

2.6 Predicting glycosylation sites

Potential N-linked glycosylation sites in the HA protein were predicted using the web-based tool

NetNGlyc 1.0 , with sites exceeding the default threshold of 0.5 considered positive.

2.7 Antigenic epitope analysis and structural modeling

The distribution of amino acid substitutions in antigenic epitopes was visualized using Chiplot (https://www.chiplot.online/) to create a cone stack layout. For structural analysis, the publicly available three-dimensional crystal structure of the BV virus HA (B/Brisbane/60/2008, PDB ID: 4FQM) was downloaded from the Protein Data Bank (https://www.rcsb.org/). Key residue differences between the vaccine strain and Guizhou BV strains were mapped onto the HA structure using PyMOL version 3.1.0 (https://www.pymol.org/). The gain or loss of a glycosylation site resulting from amino acid substitutions at position 197 was identified.

3.Results

3.1 H1N1 subtype

From 2017 to 2023, the amino acid homology of HA protein between H1N1 virus and vaccine strains at the same period maintained at a high level, with a median of 98.72% (IQR: 98.54% -99.09%). The distribution range of homology was relatively concentrated (full range: 98.00% -99.82%), indicating that the antigenicity of H1N1 virus population was highly conservative during the study period, and it was well matched with the vaccine strain. Phylogenetic analysis revealed that all circulating strains belonged to clade 6B.1, exhibiting continuous evolution. Early strains (2017-2018) were located in subclades like 6B.1A. By 2019, the viruses diversified into multiple subclades (e.g., 6B.1A.5a.), with 91.7% carrying the S183P substitution. No H1N1 viruses were detected between 2020 and 2022. All viruses from 2023 clustered in the 6B.1A.5a.2 subclade with the A/Wisconsin/588/2019 vaccine strain but accumulated multiple substitutions, including K54Q, A186T, Q189E, R259K, k308R and I418V (Figure 1). Antigenic site analysis identified specific amino acid replacements relative to different vaccine strains, with notable changes in the Sb and Ca sites. Analysis of glycosylation sites identified eight conserved locations, with a variation at position 272 in two strains leading to a new potential

glycosylation site.A complete list of all detected amino acid substitutions and glycosylation site variations is provided in Supplementary Table S1.

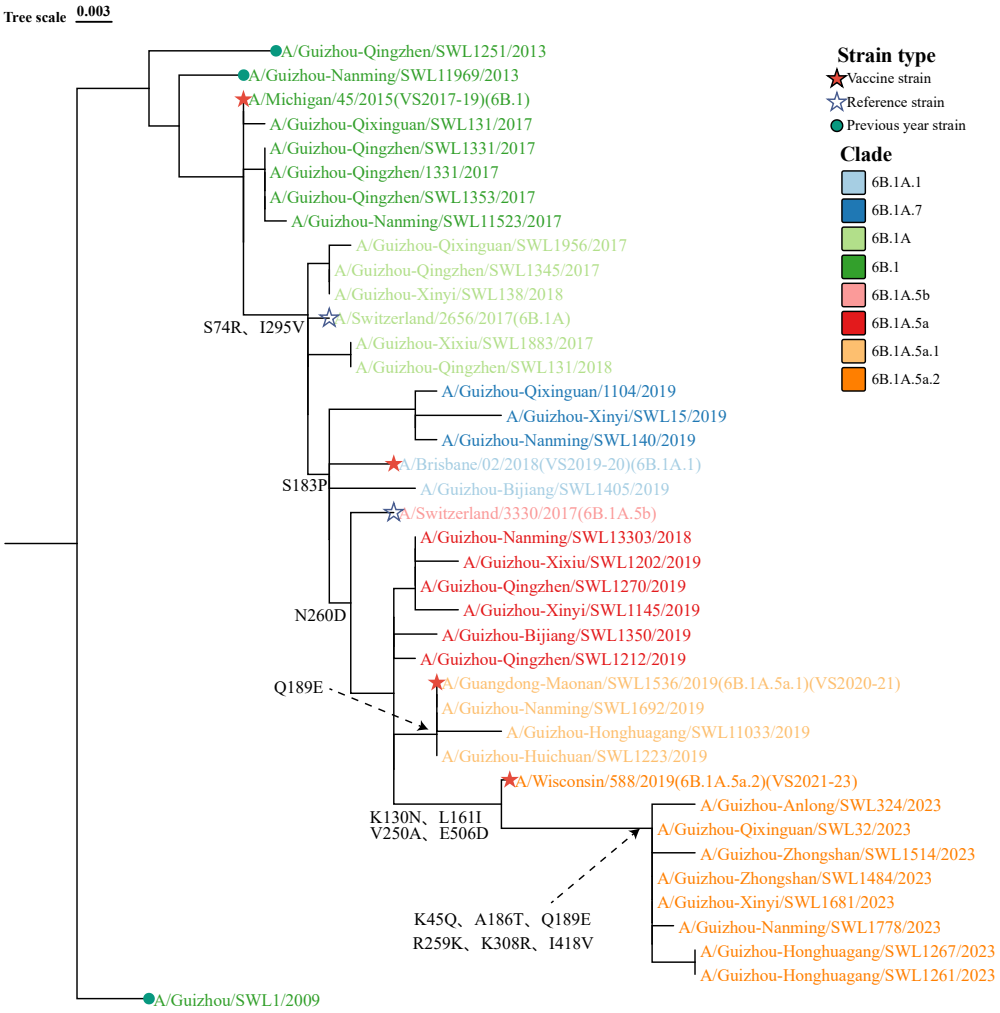


Figure 1 Phylogenetic tree of HA sequences from influenza H1N1 viruses in Guizhou Province, 2017–2023.

3.2 H3N2 subtype

In stark contrast to other subtypes, the HA amino acid homology between H3N2 virus and vaccine strain was significantly lower (median 98.00%). Most strains (IQR: 97.81% -98.00%) showed good conservation, but the homology distribution was wide (full range: 96.55% -99.09%). It is worth noting that we identified a subgroup with significantly reduced homology (n=5), and its homology with the vaccine strain was only 96.55%. Phylogenetic analysis confirmed that these low homologous strains

formed an evolutionary cluster independent of the branch of the vaccine strain, and all carried S193F mutation, which was located within the known antigenic site B, which provided a molecular explanation for their potential antigenicity differences, and also suggested that there was continuous antigenic diversity within the virus population during the study period. Phylogenetically, 88.4% of strains belonged to clade 3C.2a1. All viruses from the 2022 outbreak formed a distinct cluster within subclade 3C.2a1b.2a.1a, characterized by mutations including N159Y and N190D. By 2023, strains had further evolved into subclade 2a.3a.1. It is worth noting that there is a significant genetic distance between some vaccine strains and local epidemic strains from 2014 to 2017 (Figure 2). Antigenic site analysis revealed that a large number of key amino acid substitutions were accumulated at sites A and B (such as S198P, I192F) and receptor binding sites (such as N190D) over time. Glycosylation analysis identified key gains (e.g., at position 158 due to K160T) and losses (e.g., at position 133 due to T135K) of potential N-linked glycosylation sites, which may collectively influence viral antigenicity.A complete list of all detected amino acid substitutions and glycosylation site variations is provided in Supplementary Table S2.

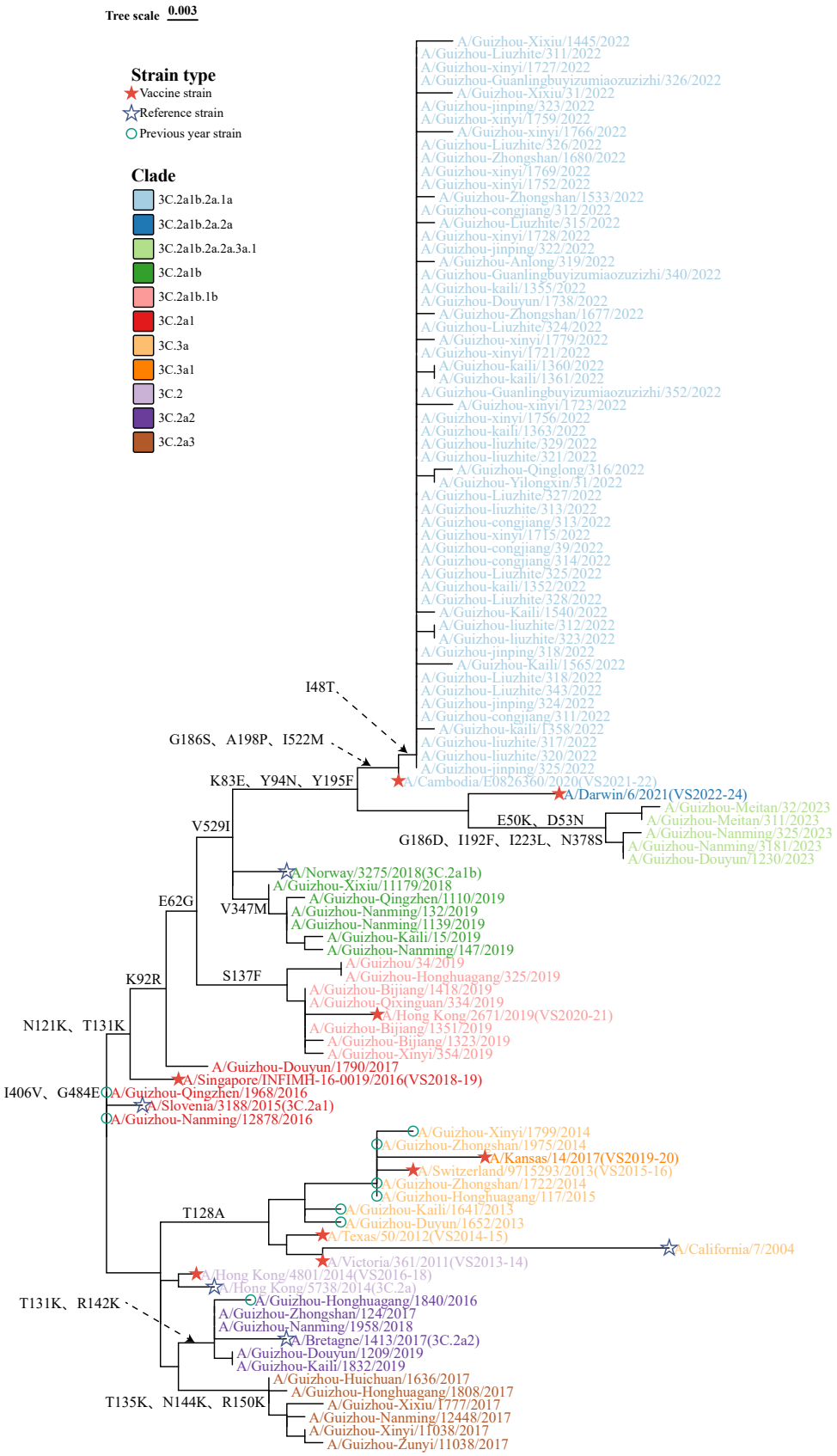
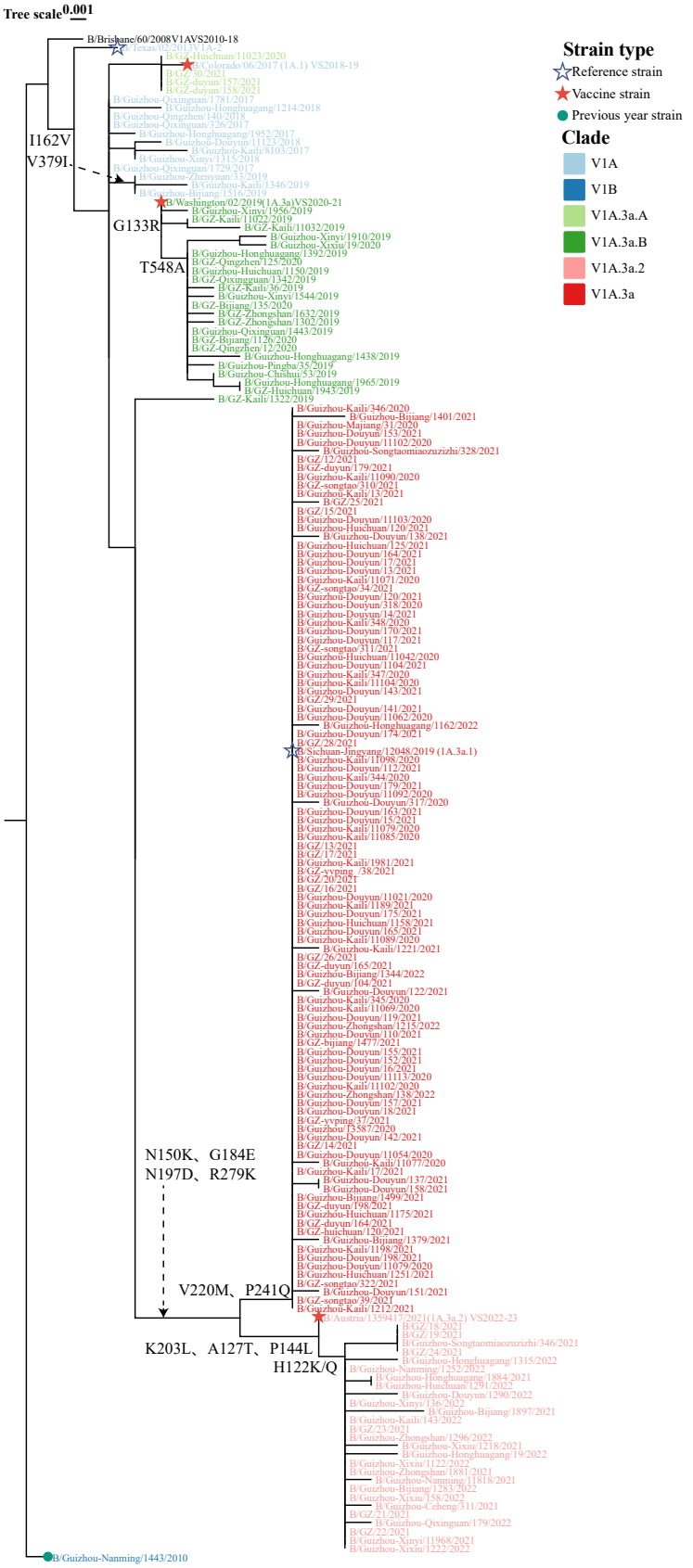


Figure 2 Phylogenetic tree of HA sequences from influenza H3N2 viruses in Guizhou Province, 2017–2023.

3.3 BV lineage

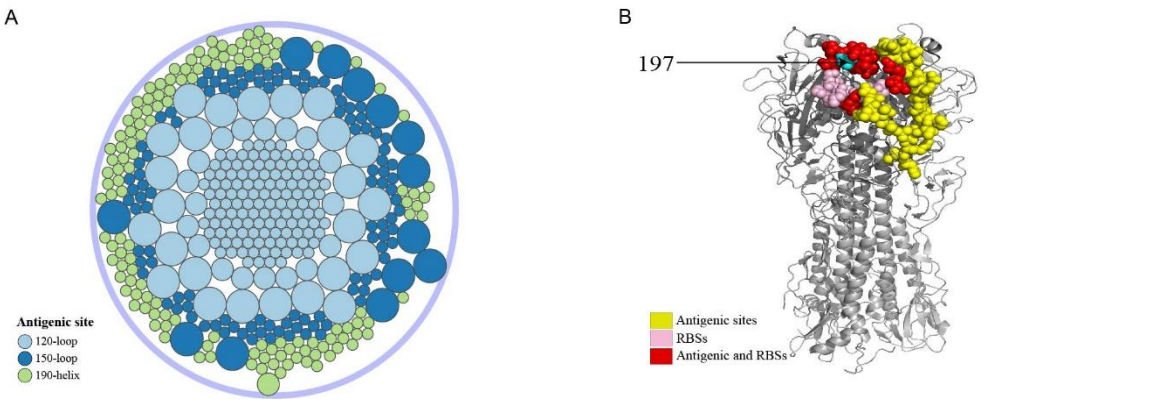
The homology between BV virus and vaccine strain was also conservative, with a median of 98.77% (IQR: 97.77% -98.94%). Despite several updates of vaccine strains, the homology distribution remained in a narrow high range (full range: 98.06% -99.82%), suggesting that the selection of vaccine strains can effectively cover the antigenic variation of BV virus. Phylogenetically, 93.0% of strains belonged to the clade 1A.3a. Notably, circulating strains in Guizhou frequently preceded vaccine updates; for instance, the 2017-2018 strains clustered with the subsequent season's vaccine strain, B/Colorado/06/2017. Since 2019, the vast majority of the strains (95.7%) have lost 162-164 amino acids. Later viruses diverged into two main subclades, 1A.3a1(with V220M/P241Q) and 1A.3a2 (with K203L/A127T/P144L) (Figure 3). Antigenic site analysis identified the 120-loop as the most variable region. Glycosylation prediction revealed dynamic gain and loss of a key glycosylation site at position 197, suggesting potential antigenic implications (Figure 4).A complete list of all detected amino acid substitutions and glycosylation site variations is provided in Supplementary Table S3.



198

199 **Figure 3 Phylogenetic tree of HA sequences from influenza BV viruses in Guizhou Province,**

200 **2017–2023.**



A. Distribution of amino acid substitutions in antigenic epitopes.

B. Mapping of amino acid substitutions and glycosylation site changes on the HA protein structure.

Figure 4 High-frequency mutations in the 120-loop and emergence of a novel glycosylation site at epitope 197 in influenza B/Victoria viruses, Guizhou, China, 2017–2023.

(A) Distribution of amino acid substitutions in antigenic epitopes. Each circle represents an individual virus strain, and the circle size indicates the number of amino acid substitutions observed at each site. (B) Three-dimensional structure of the BV virus HA protein (based on B/Brisbane/60/2008, PDB ID: 4FQM). Key residue differences between the vaccine strain and Guizhou strains are highlighted; the substitution at position 197 (arrow) indicates the gain/loss of a potential glycosylation site.

3.4 BY lineage

The median homology of BY virus was 99.65% (IQR: 99.47% -99.65%), and the distribution range was limited (full range: 99.12% -99.65%). All circulating strains in Guizhou belonged to clade Y3 and differed from the B/Phuket/3073/2013 vaccine strain by only minor changes, including L172Q and M251V (Figure 5). Antigenic site analysis identified only two substitutions (D163E and T198P) in a limited number of 2017-2019 strains. Glycosylation site prediction revealed the loss of the 196NKT site in one strain due to a T198P mutation.

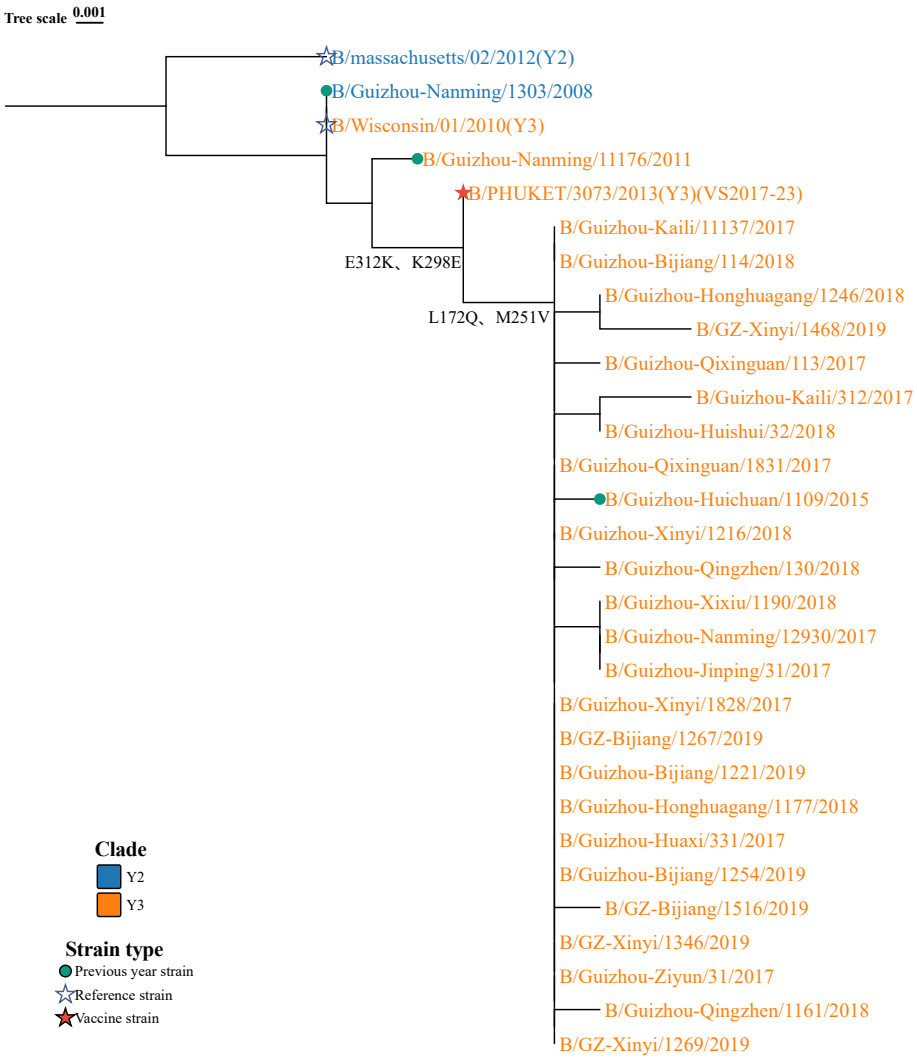


Figure 5 Phylogenetic tree of HA sequences from influenza BY viruses in Guizhou Province, 2017–2023.

4.Discussion

Seasonal influenza poses a major public health challenge, responsible for up to substantial economic losses amounting to billions of US dollars [17, 18]. In response to this challenge, this study aims to systematically analyze the genetic characteristics of hemagglutinin (HA) gene of influenza A and B viruses in Guizhou Province from 2017 to 2023, including its homology with vaccine strains, amino acid variation and glycosylation mode, in order to provide key basis for scientific prevention and

control of local influenza.

Phylogenetic analysis revealed that all H1N1 strains from Guizhou (2017-2023) belonged to clade 6B.1 and maintained high amino acid homology (median 98.72%) with vaccine strains. In 2019, the virus showed multi branch coexistence and generally carried S183P mutation, suggesting that there may be a dual drive of local pedigree revival and external new pedigree input. Antigenic site analysis identified substitutions in key epitopes, including D187A/V in the 190-helix. Notably, the evolution at position 187—from avian-preferred E187 to the swine-adapted D187—may represent a continued adaptation to human hosts. [19]Glycosylation sites remained largely stable, with only two strains acquiring a new 276NTT site due to V272F/A substitutions.

In Guizhou, H3N2 viruses from 2017-2023 predominantly belonged to clade 3C.2a1. Their significantly lower HA homology to vaccine strains, consistent with rapid evolution, was linked to frequent amino acid substitutions across antigenic sites in 2019. Notably, the K160T substitution introduced a new glycosylation site (158NYT). Variations in the 158-160 region, particularly at position 160, are known to alter antigenicity.[20] The strains of the epidemic in 2022 were all clustered in the 3c.2a1b.2a.1a subclades, indicating that the outbreak was dominated by a single input source.Viruses from 2023, evolving further, lost the 94NSS glycosylation site due to an N96S substitution, a change warranting further investigation for its potential impact on antibody recognition.

Phylogenetic analysis revealed that 93.0% of BV strains in Guizhou belonged to clade 1A.3a. Notably, some circulating strains from 2017-2019 clustered with subsequent seasonal vaccine strains (e.g., B/Colorado/06/2017, B/Washington/02/2019), indicating that local epidemiology sometimes preceded global vaccine recommendations. Antigenic analysis confirmed the 120-loop as the most variable region, with 98.24% of strains carrying substitutions. A characteristic 162-164 deletion in the 160-loop was also prevalent, this is the same finding as the influenza report issued by WHO[21]. Glycosylation analysis revealed a dynamic pattern at position 197: it was gained in 2017 via D197N but subsequently lost in 74.69% of post-2019 strains due to reverse mutations. As position 197 lies within both an antigenic site and the receptor-binding domain, its glycosylation status directly influences antigenicity, making it a critical marker for monitoring viral evolution and vaccine matching.

All BY viruses from Guizhou (2017-2019) clustered within the Y3 clade and exhibited high amino acid homology (median 99.65%) with the B/Phuket/3073/2013 vaccine strain. Antigenic analysis

identified only two substitutions (D163E and T198P) across the epitopes, which is below the established threshold for antigenic drift (≥4 changes across ≥2 epitopes)[22]. Glycosylation sites remained largely conserved, with a single strain losing the 196NKT site. These findings collectively indicate stable antigenicity of BY viruses in Guizhou, suggesting sustained effectiveness of the vaccine.

This study has two limitations:.Firstly, the lack of vaccination information of vaccinators, so it is unable to comprehensively evaluate the actual protection effect of vaccines. Secondly, although the antigenic site mutations were found, no antigenicity experiments were conducted to confirm whether these gene mutations caused antigenicity changes. Future studies should combine serological data and continuously monitor the virus.

5.Conclusion

In conclusion, our analysis of influenza viruses circulating in Guizhou Province between 2017 and 2023 reveals a high degree of HA genetic similarity (median ≥98%) to the corresponding annual influenza vaccine strains. This indicates that the seasonal vaccines have remained largely well-matched to the circulating strains during this period, providing a solid foundation for their clinical utility in preventing influenza infection in this population. Nevertheless, the detection of key mutations, even outside traditional antigenic sites, serves as an important reminder of the virus's continuous evolutionary potential. Given that annual vaccine composition relies on predicting circulating strains, these findings carry significant implications for clinical practice and public health policy: they underscore the critical need for ongoing molecular surveillance to monitor for antigenic drift, inform the timely reformulation of annual vaccines, and support clinicians in reinforcing the importance of annual vaccination as the most effective preventive measure against a constantly evolving virus.

Author contributions Yonghu Wan and Jiangfei Wang contributed equally to this work. They conceived and designed the study, carried out the experiments, and analyzed the data. Junfei Huang and Shijun Li are co-corresponding authors. They supervised the project, provided critical intellectual input, and revised the manuscript. Suye Zhao and Chunting Liu collected the clinical data and contributed to data analysis and experiments. All authors commented on previous versions of the manuscript, and all have read and approved the final manuscript.

Conflicts of Interest The authors declare no conflicts of interest.

Data Availability The datasets generated and/or analysed during the current study are available in the GenBank repository. The accession numbers for the influenza virus sequences reported in this paper are PZ152158-PZ152188, PZ152260-PZ152345, PZ152746-PZ152769, and PZ152787-PZ152957.

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Supplementary Files

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