

Methods and Applications

Systematic Evaluation and Application of Single-Nucleotide Polymorphism-Based Genotyping Methods in Varicella-Zoster Virus Molecular Epidemiology Surveillance — China, 2017–2026

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ABSTRACT

Introduction: In China, no standardized single-nucleotide polymorphism (SNP) scheme exists for varicella-zoster virus (VZV) genotyping. The 5-SNP scheme with two amplicon gene fragments lacks systematic validation. This study aimed to evaluate the accuracy and applicability of this genotyping method.

Methods: A total of 280 complete genomes were genotyped using 5 SNPs extracted from ORF22 (four SNPs) and ORF38 (one SNP) fragments. The results were compared with those of the phylogenetic clustering method as a reference. Concordance with reference was used to estimate the accuracy of the SNP scheme. The evaluated SNP scheme was applied to a national VZV surveillance screen containing 549 clinical samples from 17 Chinese provincial-level administrative divisions (2017–2026).

Results: A 97.9% concordance was observed between the 5-SNP scheme and phylogenetic clustering methods. The 2.1% discordance was mostly attributed to putative recombination and early circulation of strains from patients with herpes zoster. During the national VZV surveillance screening, 434 samples were amplified, sequenced, and genotyped using a 5-SNP scheme. Of the genotyped samples, 90.3% and 8.8% were identified as clades 2 and 5, respectively, using four ORF22 SNPs, and the remaining 0.9% as clade 4, using ORF38 SNP. All samples showed 100% intragenotypic SNP profile consistency.

Conclusion: The unified 5-SNP scheme is accurate and practical for VZV surveillance in China; however, periodic evaluation is required.

human pathogen (family *Orthoherpesviridae*, subfamily *Alphaherpesvirinae*, and genus *Varicellovirus*), causes primary varicella. Following infection, VZV establishes lifelong latency in the sensory ganglia and may reactivate, causing herpes zoster (HZ). Over 80 million varicella cases and approximately 14,000 associated deaths occur annually worldwide, and approximately 30% of patients develop HZ, imposing a substantial public health and socioeconomic burden (1–2).

According to the World Health Organization, nine distinct VZV genotypes exist (clades 1–6, 9, and provisional clades VII and VIII), each with a characteristic geographic distribution (3). Owing to the stability and size (around 125 kb) of the VZV genome, targeted single-nucleotide polymorphism (SNP) genotyping is the primary method for molecular surveillance. Various groups have published SNP schemes using clade-specific SNPs to discriminate genotypes (4). However, as more complete genomes become available and new genotypes emerge, these schemes are unsuitable for recognizing current VZV genotypes. The updated US CDC SNP scheme can differentiate all genotypes using seven SNPs from six gene fragments (ORF21+22+29+38+55+67) (5). However, amplifying the six gene fragments is labor-intensive and unsuitable for nationwide surveillance, especially in China. Therefore, a simplified 5-SNP scheme was established for small-scale surveillance in China (four in ORF22 and one in ORF 38). To date, this scheme has not been systematically evaluated, and its accuracy in identifying circulating genotypes remains unclear. In China, in addition to the predominant clade 2, minority genotypes (clades 1, 3, 4, and 5) have been identified using various schemes, making cross-comparison and communication difficult (6). Therefore, a genotyping scheme validated through nationwide surveillance is needed for consistency across research groups in China.

Varicella-zoster virus (VZV), a highly contagious

To address these gaps, the 5-SNP scheme was systematically evaluated using 280 publicly available complete genomes and nationwide surveillance data (2017–2026). This study aimed to evaluate and validate a unified genotyping approach to facilitate cross-study comparisons and reveal the dynamics of VZV genotypes in China.

METHODS

VZV Sequences Used in This Study

To extract VZV sequences from public databases (including the GenBank database and GenBase from the China National Center for Bioinformation), "Varicella and zoster virus," "VZV," and "Human alphaherpesvirus 3" were used as search terms, after which the complete genome sequences were downloaded. A total of 280 VZV sequences collected between 1964 and 2025 were downloaded for the analysis. Prototype strains of each clade were based on previously published articles (4–5).

Construction of the Phylogenetic Tree

Sequences were aligned using MAFFT (version 7.487; Osaka University, Osaka, Japan) (7). A maximum likelihood (ML) tree was constructed using IQ-TREE (version 2.3.6; Australian National University, Canberra, Australia) (8) with a general time-reversible nucleotide substitution model. Node support was evaluated using 1,000 bootstrap replicates. ML tree was visualized using the Interactive Tree of Life (version 7.6; <https://itol.embl.de/>; European Molecular Biology Laboratory, Heidelberg, Germany) (9).

Evaluation of the 5-SNP Scheme in China

Based on the consensus principles established in 2010 for VZV genotype designation, novel viral isolates were assigned to existing genotypes based on phylogenetic clustering or characteristic SNP profiles (4). Therefore, publicly available complete genomes were genotyped using phylogenetic clustering as a reference to validate SNP-based genotyping methods. All VZV genomes were aligned with the reference strain (NC001348), and SNP profiles were extracted for genotyping. Overall, concordance was observed between the SNP-based assignments and phylogenetic clustering.

VZV Surveillance and Sample Collection

Samples were collected from the National VZV Surveillance Network, which is integrated into the national measles and rubella laboratory networks. All specimens were transported in sterile containers under cold chain conditions to the National Measles Laboratory and stored at -80°C for analysis. All opportunistically collected samples from patients clinically diagnosed with varicella or HZ were used to enhance regional representation. A total of 549 samples were collected between 2017 and 2026 from local hospitals in Shandong, Liaoning, Hunan, Guangdong, Chongqing, Inner Mongolia, Qinghai, Yunnan, Henan, Gansu, Beijing, Xizang, Jilin, Jiangsu, Xinjiang, Sichuan, and Guizhou. Those provincial-level administrative divisions (PLADs) are regionally classified.

This study was approved by the Second Session of the Ethics Review Committee of the National Institute for Viral Disease Control and Prevention of China CDC. As this study involved virological surveillance aimed at public health and disease control, the ethics committee waived the requirement for informed consent.

Nucleic Acid Extraction

Viral DNA was extracted from clinical samples using the Tianlong Nucleic Acid Extraction Kit (Xi'an Tianlong Biotechnology Co., Ltd, Xi'an, China) according to the manufacturer's instructions.

RT-PCR

Real-time polymerase chain reaction (RT-PCR) was performed using a One Step PrimeScript™ RT-PCR kit (TaKaRa, Dalian, China) to identify VZV-positive samples. Information of primer and probe was described in [Supplementary Table S1](#) (available at <https://weekly.chinacdc.cn/>). The amplification conditions were as follows: 95°C for 2 min; 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1.5 min; and 72°C for 5 min.

Amplification and Sequencing

Target fragments were amplified from VZV-positive samples using a one-step PCR kit (TaKaRa, Dalian, China). The primers and amplification conditions have been described previously (10). Sequencing of the PCR products was performed by Sangon Biotech Co., Ltd. (Shanghai, China) and bidirectional sequencing was

conducted for confirmation. Sequences were assembled against the reference strain (NC001348) using Sequencher 5.0 (GeneCodes, Ann Arbor, Michigan, USA).

Genotyping

Four SNPs (positions 37902, 38055, 38081, and 38177) from 447 bp fragments of polymorphic regions in ORF22 were used. These four SNPs were used to classify clades 2 and 5. Additional clade refinement was required to differentiate clade 1 from 3, clade 6 from VIII, and clade 4 from 9 with additional SNP from ORF38 (position 69424).

RESULTS

Evaluation of the 5-SNP Scheme

A phylogenetic tree was constructed based on 280 publicly available genomes representing all recognized genotypes (except clade VII, for which no complete genome was available). Eight well-supported phylogenetic clusters corresponding to the eight established VZV genotypes were identified. Each cluster was anchored to the whole-genome sequence of the respective prototype strain and supported by a bootstrap value of 100 (Figure 1).

All sequences were genotyped using a 5-SNP scheme (Table 1). Compared with phylogenetic clustering, 97.9% (274/280) of the sequences yielded consistent results. Using this scheme, 2.1% (6/280) of the sequences could not be unambiguously assigned to a single genotype. Based on the phylogenetic references, these included three clade 3 genomes (MH709317.1, MH709346.1, and MH709375.1), one clade 4 genome (MH709316.1), one clade 5 genome (MH709324.1), and one clade 9 genome (JN704710.1) (Table 2). Three genomes (MH709317.1, MH709346.1, and MH709375.1) were reported as putative recombinant strains, whereas the other three genomes (MH709316.1, MH709324.1, and JN704710.1) were obtained from patients with HZ, indicating early circulating strains (5,11).

Application of the Evaluated Methods

This evaluation method was used for large-scale VZV surveillance. Supplementary Table S2 (available at <https://weekly.chinacdc.cn/>) summarizes the baseline characteristics of the patients. In total, 517 VZV-positive samples were identified using RT-PCR.

From these, 434 ORF22 genes were successfully amplified and sequenced. Of these, 392 (90.3%) and 38 (8.8%) SNP profiles (position 37902-38055-38081-38177) were G-C-C-A and A-T-C-G in the ORF22 gene fragments, which were identified as clades 2 and 5, respectively. Four samples (0.9%) were undetermined by ORF22 and were refined to clade 4 by adding one SNP from ORF38 (A-C-C-A-G). Although clade 2 was detected in all provincial-level administrative divisions from 2017 to 2026, clades 4 and 5 were detected only in Qinghai and Xinjiang, which are located in Northwest China. Therefore, the SNP scheme successfully genotyped all sequenced samples, illustrating the regional VZV genotype distribution (Table 3).

Clade 2 was the main genotype detected in all PLADs from 2017 to 2026, except in Qinghai, where clade 5 (35) strains outnumbered clade 2 (29) (Table 3). Clade 4 was only detected in Qinghai, accounting for 8.6% (3/35) of the sequences in 2024 and 8.3% (1/12) in 2025. Clade 5 was detected frequently in Qinghai: 50% (7/14) in 2023, 77% (27/35) in 2024, and 8.3% (1/12) in 2025. In contrast, clade 5 was sporadically detected in Xinjiang, accounting for 12.5% (3/24) of the population by 2025.

Ten varicella outbreaks were identified. All outbreaks were caused by clade 2, except for one in Qinghai, which was caused by clade 5 and represented the longest outbreak (3 months). No HZ-related outbreaks were observed (Supplementary Table S3, available at <https://weekly.chinacdc.cn/>).

DISCUSSION

In this study, the 5-SNP scheme was systematically evaluated and showed robustness, with 97.9% of publicly available genomes correctly genotyped, although it was less efficient at detecting recombinant strains (5). The SNP profile of the 5-SNP scheme from national surveillance samples showed 100% consistency within each genotype (clades 2, 4, and 5), and all samples were unambiguously genotyped, proving its practicality for large-scale VZV surveillance. Whole-genome sequencing is required to detect ambiguously genotyped strains. Although the US CDC's 7-SNP scheme effectively recognizes recombinant strains, the 5-SNP scheme is cost-effective, requiring fewer amplicons and less labor. As large-scale VZV molecular epidemiological surveillance projects (such as the National Surveillance Project for

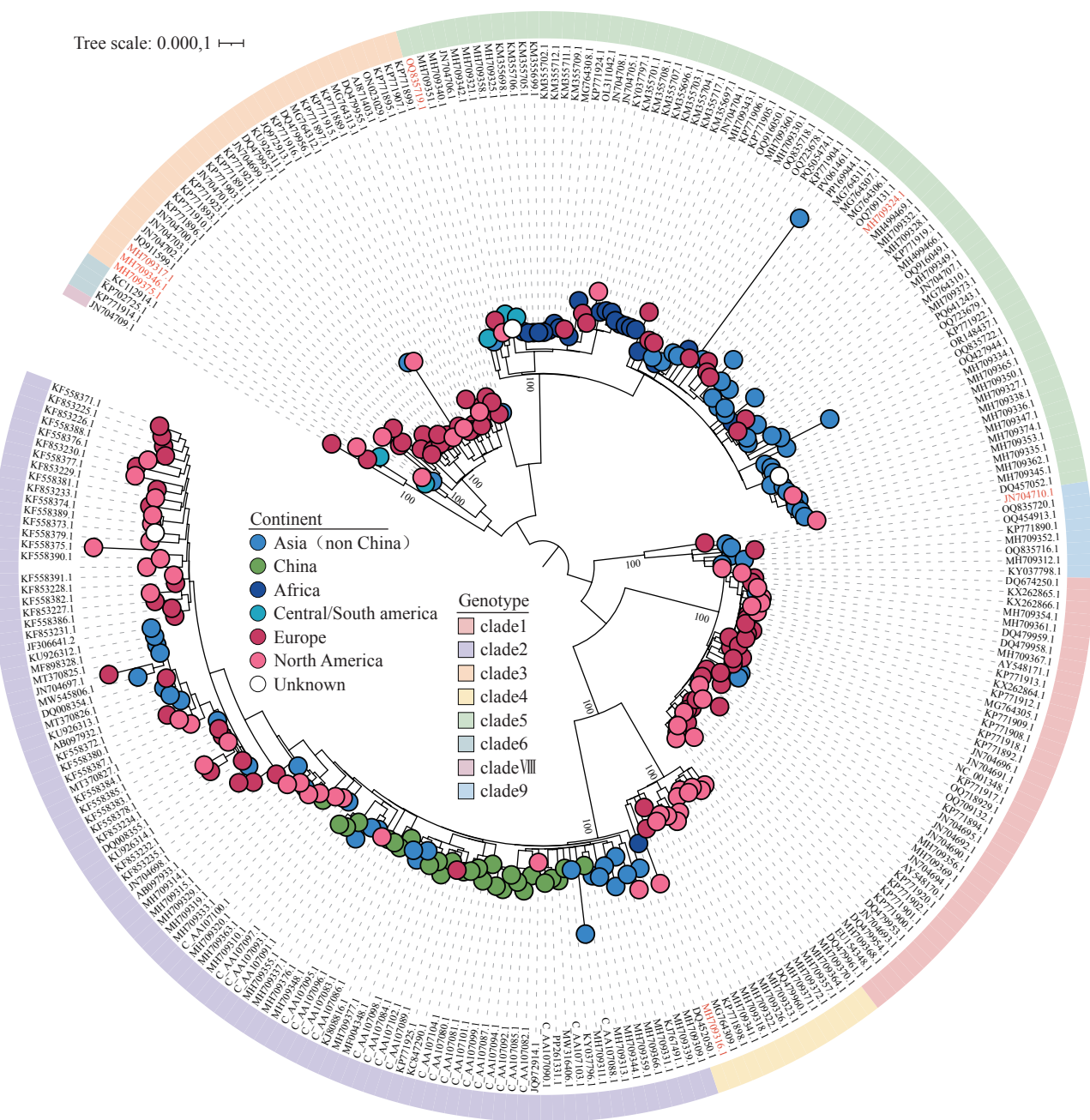


FIGURE 1. Maximum likelihood phylogenetic tree based on 280 publicly available complete varicella-zoster virus (VZV) genomes.

Note: Phylogenetic inference was tested using the bootstrap method with 1,000 replications. Bootstrap values >80% are shown. Red indicates ambiguously assigned sequence.

Febrile Rash Syndrome) are currently underway in China, the 5-SNP scheme is a practical choice for routine surveillance. Even the 7-SNP scheme failed to unambiguously genotype one genome (MH709324.1) in our analysis (Data not shown). Therefore, no perfect VZV SNP genotyping scheme exists, and all schemes should be periodically evaluated.

The 5-SNP scheme genotyped samples from seven geographical regions of China. The samples covered a

relatively broad geographic region and time span, which strengthened the representativeness of the collected samples and validated the effectiveness of our method. However, 83 samples failed to amplify despite multiple attempts using different reagents, and mutations in the primer-binding regions were excluded. Most of these samples were from a single PLAD, and failure was likely due to poor sample quality during collection or transportation (Data not

TABLE 1. Genotyping 5-SNP scheme for VZV.

SNP (position referred to NC_001348 strain)	37902	38055	38081	38177	69424
ORF	22	22	22	22	38
Clade 1	A	T	A	G	G
Clade 2	G	C	C	A	G
Clade 3	A	T	A*	G	A
Clade 4	A	C	C	A*	G
Clade 5	A	T*	C	G	G*
Clade 6	A	T	C	A	A
Clade VIII	A	T	C	A	G
Clade 9	A	C	C*	A*	A

Abbreviation: SNP=single nucleotide polymorphisms; VZV=varicella-zoster virus.

* Less common discriminating SNP.

TABLE 2. SNP profiles of public available VZV genomes genotyped using 5-SNP scheme.

Strain name	Genotype designated using phylogenetic clustering method	SNP (position referred to NC_001348 strain)				
		37902	38055	38081	38177	69424
NC 001348.1*	Clade 1	A	T	A	G	G
AY548170.1 and other 39 genomes	Clade 1	A	T	A	G	G
AB097932.1*	Clade 2	G	C	C	A	G
AB097933.1 and other 101 genomes	Clade 2	G	C	C	A	G
AJ871403.1*	Clade 3	A	T	A	G	A
DQ479955.1 and other 27 genomes	Clade 3	A	T	A	G	A
DQ452050.1*	Clade 4	A	C	C	A	G
DQ479960.1 and other 12 genomes	Clade 4	A	C	C	A	G
DQ457052.1*	Clade 5	A	T	C	G	G
JN704704.1 and other 74 genomes	Clade 5	A	T	C	G	G
KP702725.1*	Clade 6	A	T	C	A	A
KP771914.1 and KC112914.1	Clade 6	A	T	C	A	A
JN704709.1*	Clade VIII	A	T	C	A	G
KP771890.1 and other 6 genomes	Clade 9	A	C	C	A	A
MH709317.1 and other 2 genomes	Clade 3	A	T	C [†]	G	A
MH709316.1	Clade 4	A	C	C	G [†]	G
MH709324.1	Clade 5	A	C [†]	C	-	G
JN704710.1*	Clade 9	A	C	A [†]	G [†]	A

Abbreviation: SNP=single nucleotide polymorphisms; VZV=varicella-zoster virus.

* Prototype strain of each genotype.

[†] SNP mutation compared to prototype strains, except for clade 9, where the mutation exists in the prototype strain.

shown). A shift in the VZV genotype distribution (from clade 2 to clade 5) was observed in Qinghai. Therefore, with broader vaccination policies in China, genotype surveillance is vital as the VZV genotype distribution shifts as varicella and HZ incidence rates decline. This has occurred with other viruses with widespread vaccination programs, such as measles (12). In contrast, a prolonged outbreak induced by clade 5 was detected in western China. A similar trend was documented elsewhere, including a 6-month outbreak

in Guinea-Bissau (13) and 2-month multi-village outbreak in India (14), which were induced by clade 5, suggesting that it may possess enhanced transmission fitness. Clade 4 was associated with severe cases, highlighting the need for closer attention to clinical symptoms during surveillance in relevant regions (15). Therefore, monitoring the VZV genotype distribution is crucial for timely policy interventions in disease control and clinical management.

This study had certain limitations. First, the

TABLE 3. Geographic distribution, epidemiological and genotype information of obtained sequences.

Location				Collection time										Outbreak relation		Genotype		
Region	Number	PLAD	Number	2017	2018	2019	2020	2021	2022	2023	2024	2025	2026	Related	Not related	Clade 2	Clade 4	Clade 5
North China	77	Beijing	2	-	-	-	-	-	-	-	-	2	-	-	2	2	-	-
		Inner Mongolia	75	-	-	-	-	-	-	25	40	10	-	-	75	75	-	-
Northeast China	80	Liaoning	70	4	39	27	-	-	-	-	-	-	-	-	70	70	-	-
		Jilin	10	10	-	-	-	-	-	-	-	-	-	-	10	10	-	-
East China	124	Shandong	74	-	-	-	29	6	6	7	23	3	-	6	68	74	-	-
		Jiangsu	50	-	-	-	-	-	-	-	-	49	1	15	35	50	-	-
Central China	16	Henan	6	-	-	-	-	-	-	-	-	6	-	-	6	6	-	-
		Hunan	10	-	-	-	-	-	-	-	10	-	-	10	-	10	-	-
South China	10	Guangdong	10	-	-	-	-	-	-	-	10	-	-	-	10	10	-	-
Southwest China	26	Yunnan	8	-	-	-	-	-	-	-	-	8	-	8	-	8	-	-
		Xizang	2	-	-	2	-	-	-	-	-	-	-	-	2	2	-	-
		Chongqing	9	-	-	9	-	-	-	-	-	-	-	-	9	9	-	-
		Sichuan	6	-	-	4	2	-	-	-	-	-	-	-	6	6	-	-
		Guizhou	1	-	-	1	-	-	-	-	-	-	-	-	1	1	-	-
Northwest China	101	Qinghai	68	-	-	7	-	-	-	14	35	12	-	19	49	29	4	35
		Gansu	9	-	-	-	-	-	-	-	-	9	-	9	-	9	-	-
		Xinjiang	24	-	-	-	-	-	-	-	-	24	-	-	24	21	-	3
Sum	434	17	434	14	39	50	31	6	6	46	118	123	1	67	367	392	4	38

Abbreviation: PLAD=provincial-level administrative division.

“-” no sample collected

opportunistic nature of sample collection may have led to an underestimation of genotype diversity. Second, owing to the lack of specific sample information, the outbreak duration was estimated by combining the initial and final sampling dates among the same outbreak units. Future studies should incorporate a broader and more systematic sampling framework and periodic re-evaluation of SNP markers to maintain the accuracy and relevance of molecular surveillance.

In conclusion, the 5-SNP scheme genotyping method was validated as an accurate and practical tool for large-scale surveillance studies and is recommended as a unified VZV genotyping method for routine VZV molecular epidemiology surveillance in China, although periodic re-evaluation is needed.

Conflicts of interest: No conflicts of interest.

Ethical statement: Approved by the Second Session of the Ethics Review Committee of the National Institute for Viral Disease Control and Prevention of China CDC. As this study involved virological surveillance aimed at public health and disease control, the ethics committee waived the requirement for informed consent.

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SUPPLEMENTARY MATERIALS

SUPPLEMENTARY TABLE S1. Information on primer for VZV detection.

primer	Sequence 5'-3'
VZV-1	AAACCGCACATGATAACGC
VZV-2	GATTAGGACCATCCCCCG
VZV probe	ACAATGAGTAGTGGCTTTATGGCGAG

Abbreviation: VZV=Varicella-Zoster Virus.

SUPPLEMENTARY TABLE S2. Baseline characteristics of 549 patients included in this study.

Variable	n (%)
Sex, male/female/unknown	294 (53.5)/221 (40.2)/34 (6.1)
Age (years), median, IQR	15, 9–21
Diagnosis	
Varicella	540 (98.4)
Herpes Zoster	9 (1.6)
Region	
North China	133 (24.3)
Northeast China	90 (16.4)
East China	150 (27.3)
Central China	20 (3.6)
South China	10 (1.8)
Southwest China	33 (6)
Northwest China	113 (20.5)

Abbreviation: IQR=interquartile range.

SUPPLEMENTARY TABLE S3. Information of varicella outbreak induced by different genotypes.

PLAD (city)	Years (month)	Duration	Place	Sampled number	Genotype
Shandong (Binzhou)	2025 (Jun)	<1 week	Primary school	5	Clade 2
Hunan (Pingjiang)	2024 (May)	a week	Primary school	10	Clade 2
Yunnan (Pu'er)	2025 (Apr)	<1 week	Kindergarten	8	Clade 2
Gansu (Lanzhou)	2025 (Jul)	<1 week	Kindergarten	10	Clade 2
Jiangsu (Suqian)	2025 (Dec)	Unknown	Unknown	1	Clade 2
Jiangsu (Suqian)	2025 (Oct)	<1 week	Unknown	2	Clade 2
Jiangsu (Suqian)	2025 (Dec)	<1 week	Unknown	5	Clade 2
Jiangsu (Suqian)	2025 (Nov)	<1 week	Unknown	7	Clade 2
Qinghai (Yushu)	2023–24 (Dec–Jan)	1 month	Primary school	8	Clade 2
Qinghai (Yushu)	2023–24 (Dec–Mar)	3 months	High school	11	Clade 5

Abbreviation: PLAD=provincial-level administrative division.