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The resurgence of influenza A/H3N2 virus in Australia after the relaxation of COVID-19 restrictions during the 2022 season

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Abstract

This study retrospectively analyzed the genetic characteristics of influenza A H3N2 (A/H3N2) viruses circulating in New South Wales (NSW), the Australian state with the highest number of influenza cases in 2022, and explored the phylodynamics of A/H3N2 transmission within Australia during this period. Sequencing was performed on 217 archived specimens, and A/H3N2 evolution and spread within Australia were analyzed using phylogenetic and phylodynamic methods. Hemagglutinin genes of all analyzed NSW viruses belonged to subclade 3C.2a1b.2a.2 and clustered together with the 2022 vaccine strain. Complete genome analysis of NSW viruses revealed highly frequent interclade reassortments between subclades 3C.2a1b.2a.2 and 3C.2a1b.1a. The estimated earliest introduction time of the dominant subgroup 3C.2a1b.2a.2a.1 in Australia was February 22, 2022 (95% highest posterior density: December 19, 2021–March 13, 2022), following the easing of Australian travel restrictions, suggesting a possible international source. Phylogeographic analysis revealed that Victoria drove the transmission of A/H3N2 viruses across the country during this season, while NSW did not have a dominant role in viral dissemination to other regions. This study highlights the importance of continuous surveillance and genomic characterization of influenza viruses in the postpandemic era, which can inform public health decision-making and enable early detection of novel strains with pandemic potential.

KEYWORDS

A/H3N2, Australia, evolution, full genome amplicon sequencing, influenza, inter-clade reassortments, phylodynamic BEAST analysis

William Rawlinson and Matthew Scotch are the joint senior authors.

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1 | INTRODUCTION

Before the emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in December of 2019,[1] seasonal influenza caused by influenza A virus (IAV) and influenza B virus resulted in annual epidemics globally, including Australia. These influenza epidemics typically led to increased medical consultations and hospital admissions, imposing a substantial disease burden on healthcare systems and societies worldwide.[2–4] Furthermore, these epidemics resulted in an estimated 290 000 to 650 000 deaths every year, with a notable impact on high-risk groups (e.g., children below the age of 5 years and the elderly).[5]

As the escalation of the coronavirus disease 2019 (COVID-19) pandemic on a global scale started from the spring of 2020, countries worldwide implemented extensive public health and social distancing interventions, including mask-wearing, improved hygiene practices, travel restrictions, and lockdowns. These interventions, initially aimed at slowing the spread of SARS-CoV-2,[6] were also found to curb the circulation of other respiratory pathogens including seasonal influenza viruses.[6,7] As a result, global influenza activity was substantially decreased during 2020 and 2021.[8] In Australia, as one of the countries that adopted the "zero-COVID" approach, influenza was near-absent during this period, with only 37 influenza-associated deaths reported.[9–12] This number was significantly lower than those observed in pre-pandemic periods (e.g., 1183 deaths in 2017, 148 deaths in 2018, and 902 deaths in 2019).[13]

However, in 2022, with the reopening of international borders, resumption of international travel, and further relaxation of COVID-19 restrictions nationwide and globally, influenza, predominantly the A/H3N2 subtype, re-emerged in Australia.[9,14] The 2022 influenza season commenced earlier than usual and spread rapidly from April 2022, peaking in June rather than the typical peak in August, with a shorter duration than previous years.[10,15] A total of 225 332 notifications of laboratory-confirmed influenza were reported in the 2022 season in Australia, which was significantly higher than the 5-year average in prepandemic periods (2015–2019: 163 015).[10,15] Of these notifications, the state of New South Wales (NSW) accounted for 50.4% (113 568/225 332) of the national total.[15] It is well-recognized that the H3N2 subtype exhibits a higher mutation rate compared to other subtypes, with the appearance of new antigenic variants every 3–5 years.[16] These circumstances provided a unique setting to study A/H3N2 viruses that circulated in NSW in 2022. Furthermore, although previous studies have shown the global migration pattern of IAVs, our comprehension of the transmission dynamics specific to the A/H3N2 virus within Australia, particularly in the post-pandemic era, remains limited.[17,18]

This study aimed to retrospectively investigate the genetic characteristics of re-emerged A/H3N2 viruses that prevailed in NSW in 2022 and explore the spatial-temporal transmission dynamics of A/H3N2 viruses within Australia, as well as the role of NSW in the transmission of A/H3N2 viruses during this season.

2 | METHODS

2.1 | Clinical specimens

This study included 217 archived nasopharyngeal/throat/nasal swab specimens that had tested positive for influenza A/H3 virus using the Allplex™ Respiratory Panel 1 assay (Seegene Inc.) with cycle threshold (C_t) values < 28. These randomly selected specimens were archived from patients exhibiting symptoms of influenza-like illness (ILI) at Prince of Wales Hospital and Sydney Children's Hospital in NSW in the period spanning April–August 2022. The diagnostic real-time reverse transcriptase-polymerase chain reaction (qRT-PCR) tests mentioned above were performed on the CFX-96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc.) at the NSW Health Pathology East Serology and Virology Division. Archived specimens were then stored at −80°C until being transferred to the virology research laboratory for further analysis.

2.2 | RNA extraction and multisegment amplification by RT-PCR

Viral RNA was extracted from 140 µL of each selected influenza-positive specimen (n = 217) using the QIAamp Viral RNA Mini Kit (Cat. No. 52906, Qiagen Inc.) following the manufacturer's instructions and eluted in 50 µL of nuclease-free water. The complete genome of IAV was amplified using a single multisegment RT-PCR reaction with the Uni/Inf primer set as outlined in previous studies.[19,20] The 50 µL reaction mixture consisted of 25 µL of 2× RT-PCR buffer, 0.4 µL of Uni12/Inf1 (10 µM), 0.6 µL of Uni12/Inf3 (10 µM), 1 µL of Uni13/Inf1 (10 µM), 17 µL of nuclease-free water, 1 µL of SuperScript III Platinum Taq High Fidelity DNA Polymerase (Cat No. 12574035, Invitrogen™), and 5 µL of extracted RNA. The amplification was performed on a SimpliAmp™ thermal cycler (Thermo Fisher Scientific), with thermocycling conditions detailed previously.[20]

2.3 | Next-generation sequencing and sequence assembly

Amplicon libraries for short-read sequencing were then prepared using the Illumina DNA Prep Kit (Illumina) with the Illumina DNA/RNA UD Indexes (Illumina) according to the manufacturer's instructions. Sequencing was performed on the MiSeq (Illumina) (details in Supporting Information).[20] The resulting sequencing reads were quality checked using FastQC (v0.11.9) and then trimmed using fastp (v0.23.0) to remove low-quality and short reads.[21,22] Clean reads were assembled using the FLU module of the Iterative Refinement Meta-Assembler (IRMA, v1.0.2) pipeline with default settings.[23] The raw sequencing reads were deposited in the National Center for Biotechnology Information Sequence Read Archive database (Bioproject: PRJNA1101853). Consensus nucleotide sequences were deposited in GenBank (Supporting Information S2: Table S1).

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2.4 | Phylogenetic analysis

Phylogenetic analysis was conducted using nucleotide sequences of the hemagglutinin (HA) genes from A/H3N2 viruses detected from NSW. Reference sequences were obtained from the Global Initiative on Sharing All Influenza Data (GISAID) database, including: (1) 215 representative strains reported from other regions of Australia and 13 nearby Asia/Pacific countries (Bangladesh, Cambodia, China, Indonesia, Japan, South Korea, Malaysia, New Caledonia, New Zealand, Philippines, Singapore, Thailand, and Vanuatu) during the same sampling period (April–August 2022), selected using the Nextstrain Augur filter command (Supporting Information S2: Table S2); (2) vaccine strains (egg-based) recommended for southern hemisphere (SH) influenza seasons (specifically A/Darwin/9/2021 for 2022, A/Hong Kong/2671/2019 for 2021, and A/South Australia (SA)/34/2019 for 2020); and (3) clade-defining sequences as indicated on the Nextstrain platform.[24–28] Sequence alignment was performed using the Augur align command (MAFFT method with default parameters).[24] The phylogenetic tree of HA gene was constructed using the maximum likelihood method via the Augur pipeline (IQ-TREE2 tool with default settings)[24] and then visualized using the R package ggtree.[29] Moreover, MEGA 11 software was used to translate nucleotide sequences to amino acid sequences and identify amino acid substitutions within the HA protein of study viruses in comparison to the 2022 vaccine strain (A/Darwin/9/2021), particularly at major antigenic sites.[30] These substitutions were numbered based on the H3 numbering system.

2.5 | Phylodynamics of seasonal influenza A/H3N2 in Australia

The HA sequences of representative NSW strains identified in this study, along with additional strains from other Australian states/territories available on GISAID, were included in this analysis (see details in the Supporting Information S1 document and accession numbers are listed in Supporting Information S2: Table S3). The temporal structure of the sequence data was evaluated using TempEst.[31]

In BEAUti, an HKY + G nucleotide substitution model and a nonparametric Bayesian skygrid coalescent tree prior was selected.[32] We employed a Bayesian stochastic search variable selection model with an asymmetric discrete-trait approach on BEAST v1.10.4 to infer the state-to-state A/H3N2 virus spread in Australia[33] and recorded the history of the Markov jump counts between discrete states. In addition, a strict clock model was favored over a relaxed molecular clock model, as the posterior coefficient of variation distribution as shown in Tracer v1.6 is at or near zero.[34,35] Two Markov Chain Monte Carlo chains were run independently for 500 million generations and sampled every 50 000 steps. Adequate mixing and convergence were confirmed by the effective sample size of at least 200 using Tracer v1.6.[35] After discarding the first 10% of each run as burn-in, two runs were combined using LogCombiner v1.10.4.[33] The time-scaled maximum clade credibility (MCC) tree was generated using TreeAnnotator v1.10.4 and visualized using the R package ggtree.[36]

TreeMarkovJumpHistoryAnalyzer from a prerelease version of BEAST v1.10.5 was used to collect the location and timing of each Markov jump from the posterior tree distribution annotated with Markov jumps histories.[37,38] The output generated from TreeMarkovJumpHistoryAnalyzer was analyzed and then visualized in the chord diagram in R to show viral migrations between the states/territories in Australia. SpreaD3 v0.9.6 was used to calculate Bayes Factor (BF), to confirm the most parsimonious origin-destination scenarios.[39] Furthermore, the mean evolutionary rate of the HA gene of Australian A/H3N2 viruses was analyzed using the Bayesian statistical inference approach implemented in BEAST v1.10.4.

2.6 | Interclade reassortment analysis

To identify putative interclade reassortment events, phylogenetic trees for all eight influenza segments were constructed using IQ-TREE (v2.2.0).[40] The best-fitted model was selected using ModelFinder, integrated within the IQ-TREE software, based on the Bayesian Information Criterion.[41] Complete reference genomes were also retrieved from the GISAID platform and were listed in Supporting Information S2: Table S4. The resulting trees were visualized using FigTree (v1.4.4).[36]

2.7 | Amino acid sequence analysis

Nucleotide sequences of non-HA genes were translated into amino acid sequences in MEGA 11 (details in Supporting Information). The translated sequences were compared to the 2022 vaccine strains to examine amino acid changes and screen them for known genetic markers associated with genotypic antiviral drug susceptibility and increased virulence.

2.8 | Ethics statement

The study was approved by the Sydney Children's Hospitals Network (SCHN) Human Research Ethics Committee (ETH02051).

3 | RESULTS

3.1 | Sample characteristics

The next-generation sequencing was performed on 217 selected respiratory specimens (from 217 patients). Of these specimens, complete genomes of 216 specimens (99.5%) were obtained. The remaining specimen was successfully sequenced for most of viral genome segments (1–7). Table 1 shows the demographic

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characteristics and specimen types of these samples. The median age of the patients was 17 years, ranging from 2 months to 102 years of age. Over half (119/217) of the A/H3N2-positive specimens were from patients under the age of 19 years. Among these, the age group 10–19 years represented the highest proportion (56/217, 25.8%), followed by the group under 5 years old (37/217, 17.1%) and the group aged 5–9 years (26/217, 12.0%). In addition, most of the respiratory specimens (170/217, 78.3%) were collected from patients who visited the emergency departments and thus likely had more severe symptoms than those who did not visit the emergency department (Table 1).

TABLE 1 Characteristics of influenza-positive (successfully sequenced) specimens collected in NSW, Australia, in the 2022 influenza season.

Characteristics	*N* (% of total)
Gender	
Female	107 (49.3)
Male	110 (50.7)
Age group (years)	
<5	37 (17.1)
5–9	26 (12)
10–19	56 (25.8)
20–44	55 (25.3)
45–64	21 (9.7)
≥65	22 (10.1)
Sample type	
Nasopharyngeal swab	126 (58.1)
Throat swab	57 (26.3)
Nose swab	34 (15.6)
Sample collection month	
April	18 (8.3)
May	55 (25.3)
June	126 (58.1)
July	15 (6.9)
August	3 (1.4)
Ward of the hospitals where received cases	
Emergency department	170 (78.3)
COVID and flu assessment unit	27 (12.4)
Fever clinic	16 (7.4)
Pediatric ward	3 (1.4)
COVID ward	1 (0.5)

Note: Patients from pediatric and COVID wards were as hospitalized patients.

Abbreviations: COVID, coronavirus disease; NSW, New South Wales.

3.2 | Phylogenetic analysis

The phylogenetic analysis of the HA gene sequences was conducted to determine genetic relationships between NSW viruses reported in this study, viruses circulating in 13 nearby countries (listed in Section 2), as well as the vaccine strain for the 2022 SH influenza season.

The HA genes of all A/H3N2 viruses analyzed in this study fell into the genetic subclade 3C.2a1b.2a.2 (referred to as 2a.2), characterized by shared HA1 substitutions including Y159N, T160I (leads to the loss of glycosylation site), L164Q, G186D, D190N, and Y195F (Figure 1). These viruses clustered with A/H3N2 viruses detected in other regions of Australia and most neighboring Asian and Oceanian countries during the same period, with the exception of China (where all strains belonged to the subclade 3C.2a1b.2a.1 (referred to as 2a.1)). The vaccine strain (A/Darwin/9/2021) also belonged to the same subclade 3C.2a1b.2a.2 and shared the HA1 substitution H156S with NSW viruses and reference viruses from neighboring Asian and Oceanian countries. Further analysis using clade-defining sequences revealed that NSW viruses could be further classified into two subgroups, 2a.2a.1 (213/217, 98.2%) and 2a.2a.3 (4/217, 1.8%). NSW subgroup 2a.2a.1 viruses were closely related to viruses from elsewhere in Australia, as well as Cambodia, Korea, Singapore, Thailand, Vanuatu, and New Zealand, whereas NSW subgroup 2a.2a.3 viruses were closely related to viruses from Bangladesh and New Caledonia. During the study period, the subgroup 2a.2a.1 viruses were predominant within the southern temperate zones (Figure 1).

The comparison of HA sequences between all NSW strains of this study and the 2022 SH vaccine strain (A/Darwin/9/2021) revealed amino acid similarities ranging from 98.59% to 99.29% for subgroup 2a.2a.1 viruses, and from 98.59% to 98.94% for subgroup 2a.2a.3 viruses. Additionally, five consistent amino acid substitutions were observed in predominated 3C.2a1b.2a.2a.1 viruses compared to the vaccine strain and six consistent amino acid substitutions were observed in 3C.2a1b.2a.2a.3 viruses (Table 2). Among these substitutions, D53G/N (Site C), N96S (Site D), and N186D (Site B) were located within known antigenic sites.[42,43]

3.2.1 | Phylodynamic analysis

Bayesian phylodynamic analysis revealed the temporal introduction of the predominated subgroup 2a.2a.1 virus into Australia during the 2022 influenza season and its subsequent spread within the country, as visualized from an MCC tree (Figure 2). We estimate the earliest introduction of this subgroup virus into Australia (via the state of Victoria) was February 23, 2022 (95% highest posterior density [HPD]: December 19, 2021–March 13, 2022). Based on Markov jump analysis, Victoria was the pivotal location for driving transmission of the subgroup 2a.2a.1 across the country. Western Australia (WA) showed a different pattern of viral inflows compared to other states/territories. It was observed that WA received viral introductions from

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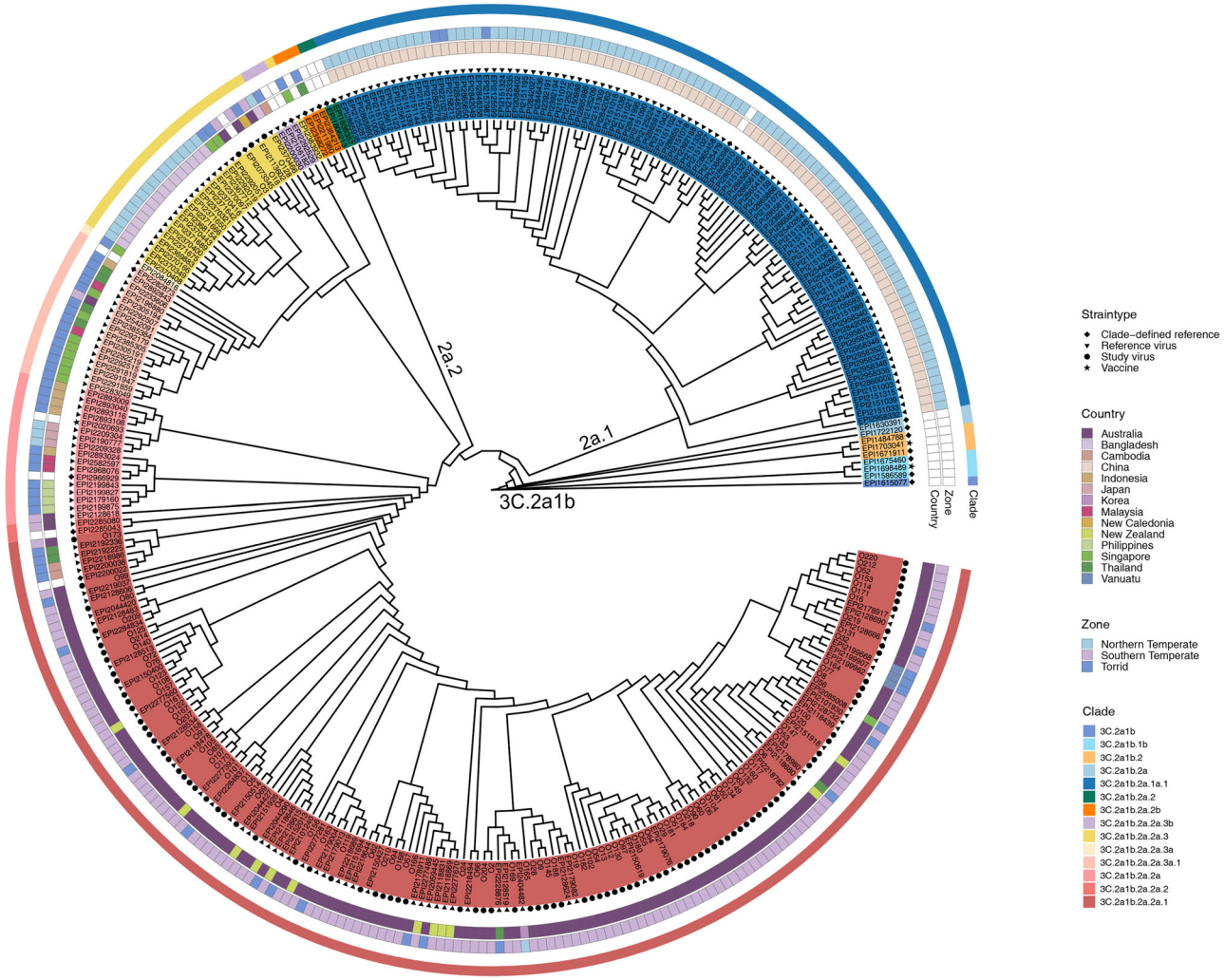

FIGURE 1 The phylogenetic tree was inferred from 328 hemagglutinin (HA) gene sequences including the New South Wales (NSW) A/H3N2 viruses detected in this study (a single strain was kept as representative of identical sequences/each region). This season, A/Darwin/9/2021 (egg-based) was suggested as the vaccine strain for southern hemisphere (SH). The tree was constructed by the maximum likelihood method using the Augur pipeline. The tree was rooted using the oldest sequence in the data set.

TABLE 2 List of amino acid substitutions differences in HA genes between 2022 SH vaccine strain and NSW 2022A/H3N2 viruses.

Amino acid changes in HA						
A/H3N2 3C.2a1b.2a.2a.1 sequences (*n* = 213) compared to A/Darwin/9/2021						
HA	D53G (C)	D104G	N186D (B)	G225D	K276R	
A/H3N2 3C.2a1b.2a.2a.3 sequences (*n* = 4) compared to A/Darwin/9/2021						
HA	D53N (C)	N96S (D)	N186D (B)	I192F	G225D	N378S

Note: (1) Only amino acid substitutions found in all sequences in each genetic clade are presented in this table. (2) The amino acid numbering is counted without the signal peptide. (3) B, C, and D in parentheses represent known antigenic sites of A/H3N2 viruses.

Abbreviations: HA, hemagglutinin; NSW, New South Wales; SH, southern hemisphere.

multiple states/territories, including Australian Capital Territory, Queensland, SA, and Victoria. Interestingly, NSW, despite being the most populous state, did not appear to play a significant role for viral dissemination to other states/territories during this season (Victoria was the primary source of the viral migration flow into NSW). These identified transmission routes were also supported by the BF (BF > 3) calculated values (Supporting Information S2: Table S6). The mean evolutionary rate of the HA gene of these Australian viruses was estimated to be 4.19×10^{-3} (95% HPD = 3.15×10^{-3}–5.19×10^{-3}) nucleotide substitutions per site per year.

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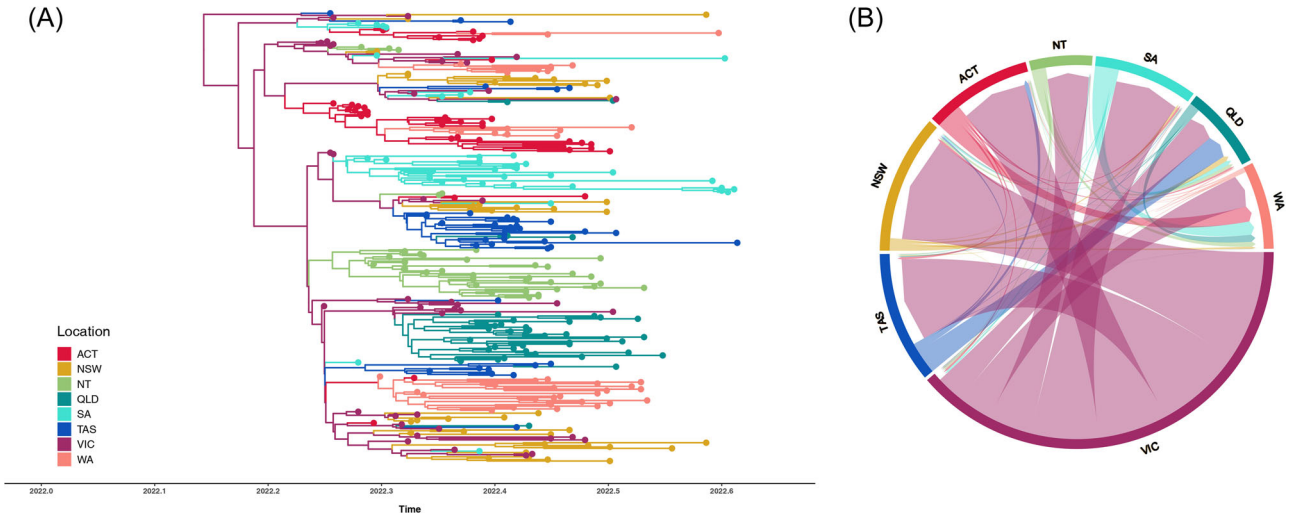

FIGURE 2 Phylogeographic estimates of A/H3N2 spread within the states of Australia. (A) Time-scaled maximum clade credibility (MCC) tree of hemagglutinin (HA) sequences of the predominated subgroup 2a.2a.1A/H3N2 viruses during the 2022 influenza season. Each branch is colored according to the geographic region. (B) The chord diagram is based on the posterior expectations of the Markov jumps between states/territories in Australia. In this visualization, the viral migration flow originating from a specific location begins near the outer ring and terminates with an arrowhead positioned farther from the destination location, depicting the directional movement between the states/territories. The colors for A and B represent the states/territories depicted in the legend. ACT, Australian Capital Territory; NSW, New South Wales; NT, Northen Territory; QLD, Queensland; SA, South Australia; TAS, Tasmania; VIC, Victoria; WA, Western Australia.

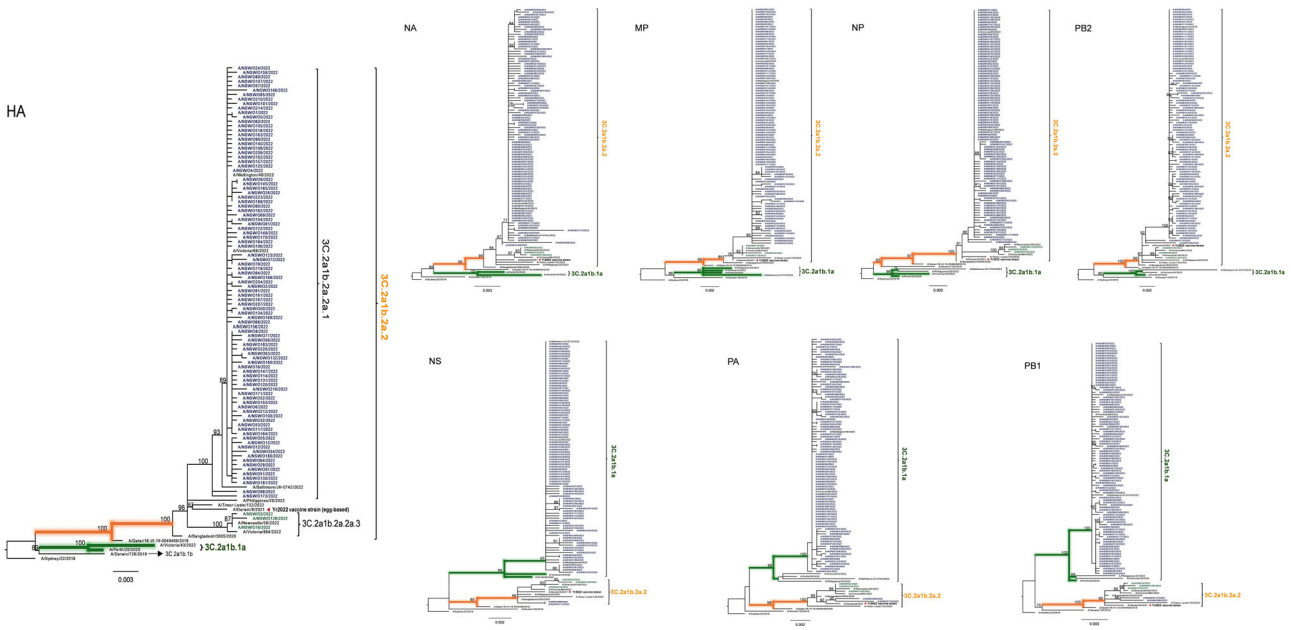

FIGURE 3 Phylogenetic trees of all gene segments (PB2, PB1, PA, HA, NP, NA, M, and NS) of A/H3N2 strains identified in this study. Reference sequences from the platform Global Initiative on Sharing All Influenza Data (GISAID) were included in the trees. Only representative New South Wales (NSW) strains are shown on the phylogenetic trees (identical genomes were removed). These representative strains are color-coded by subgroups: blue for 2a.2a.1 and green for 2a.2a.3. Main branches corresponding to subclades 3C.2a1b.2a.2 and 3C.2a1b.1a are highlighted in orange and green, respectively. Trees were constructed using IQ-TREE via the maximum likelihood method with bootstrap values for 1000 replicates. Only bootstrap values > 70% are displayed.

3.3 | Interclade reassortment identification

A total of 216 complete NSW H3N2 genomes were included in this analysis (Supporting Information S2: Table S5). The phylogenetic trees of all segments of these NSW viruses revealed that some of them were classified into the subclade 2a.2 in the HA gene, while their internal gene segments (NS, PA, and PB1) clustered with reference viruses from the subclade 1a, suggesting probable interclade

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reassortment events (Figure 3). Among the NSW genomes analyzed, 210 genomes (97.2%; 210/216) were identified as probable interclade reassortments. One type of reassortments was observed in NSW viruses, namely 5:3 (the probable reassortment events occurred in PB1, PA, and NS segments) (Supporting Information S2: Table S5). These A/H3N2 viruses with probable interclade reassortment events were detected across all age groups within our patient cohort (Supporting Information S2: Table S5).

3.4 | Genotypic antiviral drug susceptibility

None of the NSW viruses harbored amino acid substitutions known to confer reduced susceptibility to neuraminidase inhibitors (NAIs) or cap-dependent endonuclease inhibitor (CENI; Baloxavir), such as E119V, Q136K, D151E, R292K, and N294S in the NA protein, and I38T in the PA protein.[20,44]

4 | DISCUSSION

We investigated the genetic characteristics of A/H3N2 viruses that circulated in NSW during the 2022 season and provided additional insight into the spatiotemporal pattern of the dominated subgroup of A/H3N2 viruses within Australia during this period. Phylogenetic inference revealed that all NSW A/H3N2 viruses belonged to the genetic subclade 3C.2a1b.2a.2. This was the predominant subclade among A/H3N2 viruses that circulated in most countries worldwide during the 2021–2022 season, except for China and Timor-Leste. It maintained its global dominance during the subsequent 2022–2023 season.[45–48] Within the subclade 3C.2a1b.2a.2, NSW viruses further diversified into two genetic subgroups: most viruses belonged to subgroup 2a.2a.1 and carried additional HA1 amino acid substitutions D53G, D104G, and K276R. The remaining minority of viruses were classified into subgroup 2a.2a.3 and harbored additional HA1 amino acid substitutions D53N, N96S, and I192F. Based on available sequences deposited in GISAID, the dominant subgroup 2a.2a.1 had not been reported globally before 2020. It was first reported in the United States in July 2021, with subsequent detections documented in Europe and Asia. However, two published studies indicate that although this subgroup was observed in the United States and Europe during 2021, it constituted only a minority of circulating A/H3N2 viruses in these regions.[47,49] In Australia, a very small number of cases ($n = 6$) belonging to this subgroup were first reported in December 2021. Additionally, it was observed that NSW viruses clustered together with viruses from other Australian regions, as well as neighboring Southeast Asian and Oceanian countries during the same period, suggesting the possible interrelation of A/H3N2 viruses between Australia and nearby Southeast Asian and Oceanian countries. These viruses and the corresponding vaccine strain also shared the amino acid substitution H156S in the HA1 protein, which is located within the antigenic site B and the receptor binding site.[50,51] The previous studies have mentioned that the mutation occurred at this position may impact receptor binding and viral replication fitness.[50,52]

Our findings show NSW viruses were of the same HA genetic subclade (2a.2) as the corresponding 2022 vaccine virus (A/Darwin/9/2021), indicating genetically similarity between the investigated viruses and the vaccine strain. However, we observed that NSW viruses harbored several additional substitutions in HA protein compared to the vaccine-like virus, with four substitutions located within major antigenic sites B (N186D), C (D53G/N), and D (N96S). Despite these amino acid variations, there was still a significant reduction in hospitalization risk due to the influenza vaccine effectiveness of 44% (95% confidence interval: 22%–60%) for that season.[53]

We did not identify known genetic markers associated with antiviral resistance (for either NAIs or CENI) in NSW viruses. This finding supports the continued clinical use of these antiviral drugs for the treatment of influenza infections at the study site. Furthermore, multiple consistent amino acid substitutions were identified in non-HA proteins when compared to the vaccine strain (Supporting Information S2: Table S7). However, the functional significance of these substitutions remains unknown and warrants further investigation.

After nearly 2 years of pandemic-related border closures, Australia first eased travel restrictions for some fully vaccinated groups (e.g., international students) in November 2021, and subsequently fully reopened international borders to all COVID-19 vaccinated travelers on February 21, 2022.[54,55] Interestingly, our analysis estimated that the dominant subgroup 2a.2a.1 first entered Australia on February 23, 2022 (95% HPD: December 19, 2021–March 13, 2022), coinciding with the period of relaxed travel restrictions. This timing also aligns with data from the Australian Bureau of Statistics, which reported a significant increase in international arrivals in the months immediately following November 2021.[56] These findings suggest that this dominant viral subgroup may have been introduced into Australia following the easing of travel restrictions, potentially from an international source, and then spread within the nation. Moreover, our study identified possible transmission routes of the dominant subgroup 2a.2a.1 between states and territories in Australia. Consistent with a previous study that investigated four severe influenza seasons caused by A/H3N2 viruses in Australia (2003, 2007, 2012, and 2017), our finding also showed the linking of transmission between Victoria and other states/territories.[57] However, our analysis employed an asymmetric model in discrete phylodynamic analysis to reveal that Victoria was the source location for viral transmission to other states and territories, rather than being the recipient state for transmission from other regions during this season.[57] Additionally, in contrast to previous A/H3N2 seasons,[57] we did not observe NSW playing a significant role in the dissemination of A/H3N2 viruses to other regions, suggesting a change in transmission pattern across different seasons. Given that Victoria and NSW are the most populous states in Australia and the top destinations for both domestic and international travelers, future studies are warranted to better understand state-to-state influenza virus transmission patterns, as well as international source and states. These findings will provide valuable information for influenza epidemic control in Australia, such as travel recommendations and adjustments in public health measures.

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The evolutionary analysis further revealed that the mean evolutionary rate for the HA gene of Australian A/H3N2 viruses (including NSW viruses) was estimated as 4.19×10^{-3} (95% HPD = 3.15×10^{-3}–5.19×10^{-3}) nucleotide substitutions per site per year. This estimation is similar to the mean evolutionary rates (3.37×10^{-3}–4.84×10^{-3}) reported in other countries in the pre-pandemic era.[58,59] One possible explanation is that the environment in Australia after the pandemic might have created selective conditions similar to those existing in other areas before the pandemic. This similarity might stem from changes in factors like population immunity, healthcare practices, or the dynamics of viral transmission. Furthermore, the reintroduction of regular seasonal influenza patterns after the pandemic might have exerted evolutionary pressures similar to those seen before the pandemic. However, more research is needed to investigate different influencing factors during these periods.

Interclade reassortment events among A/H3N2 viruses were commonly observed in this study (210/216 genomes), with all classified as the reassortment type 5:3, involving HA/NA/PB2/NP/M segments from the subclade 2a.2 and the PB1/PA/NS segments from the subclade 1a. Similar interclade reassortment events have also been documented during the 2022-2023 season in both Canada and the United States, although the specifics are slightly different.[51,60] These observations may suggest that interclade reassortment events are relatively common among A/H3N2 viruses that circulated in the 2022/2023 season. Additionally, interclade reassortments involving NA and other gene segments have been also reported in previous influenza seasons, such as between the clades 3C.2a2 and 3C.2a1a during 2017–2018 season[61] and between the clades 3C.3a1 and 3C.2a during 2018–2019 season.[46] However, the significance of A/H3N2 reassortment on clinical severity remains unclear, and future studies integrating patient clinical data with whole genomic information would help elucidate the potential impact of A/H3N2 viruses with interclade reassortments on disease severity.

This study had several limitations. The interpretation of our results was limited to genotypic characteristics. However, antigenic analyses of 2022A/H3N2 viruses with and without the HA1 H156S substitution, conducted by the WHO, can complement our findings, and suggest that NSW viruses share antigenic similarity with the corresponding vaccine strain.[48] Our results should be interpreted with caution due to the limited sample size analyzed in this study, which only included patients with ILI, who sought medical consultation/treatment at two hospitals. Additionally, this study did not include any international sequences into the BEAST analysis and thus our model only assumed a single introduction of the virus into the country. If additional international sequences are included, the results might be altered. Lastly, as this is a retrospective study and there were limitations on the sampling locations, we were unable to identify the local routes of A/H3N2 transmission within the state of NSW. However, by combining our sequence data with available sequence data from GISAID and utilizing the relevant geographical information (state level), we uncovered the transmission pattern of the dominant genetic subgroup of A/H3N2 viruses within Australia and estimated its earliest introduction time into the country.

5 | CONCLUSION

The present study has outlined the genetic characteristics of influenza A/H3N2 viruses that circulated in NSW during the 2022 season and revealed a high frequency of probable inter-clade reassortment events among these NSW viruses. Our results also suggest that the emergence of the dominant subgroup 3C.2a1b.2a.2a.1 may be attributed to an international source following the easing of Australian travel restrictions. Victoria was identified as a source driving the transmission of A/H3N2 viruses in this season, and other states/territories acted as transmission "sinks." These findings underscore the importance of continuous monitoring of influenza viruses for the timely detection of novel strains and reassortment events, as well as for enhancing our understanding of influenza transmission patterns in the post-pandemic era, thereby informing public health responses.

AUTHOR CONTRIBUTIONS

Conceptualization: Xinye Wang, Matthew Scotch, and William D. Rawlinson. *Methodology*: Xinye Wang and Matthew Scotch. *Sample processing and NGS experiments*: Xinye Wang. *Formal data analysis*: Xinye Wang and Matthew Scotch. *Writing—review and editing*: William D. Rawlinson, Matthew Scotch, Ki W. Kim, Gregory Walker, and Sacha Stelzer-Braid. supervision and joint senior authors, Matthew Scotch, and William D. Rawlinson.

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CONFLICT OF INTEREST STATEMENT

The authors declare no conflict of interest.

DATA AVAILABILITY STATEMENT

All data generated during this study are contained within this manuscript and its Supporting Information files. The genome data were also made publicly available in the GenBank database.

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