

## Annual reports

# INFLUENZA VIRUSES RECEIVED AND TESTED BY THE MELBOURNE WHO COLLABORATING CENTRE FOR REFERENCE AND RESEARCH ON INFLUENZA ANNUAL REPORT, 2014

Sheena G Sullivan, Michelle K Chow, Ian G Barr, Anne Kelso

### Abstract

The WHO Collaborating Centre for Reference and Research on Influenza in Melbourne is part of the World Health Organization's (WHO) Global Influenza Surveillance and Response System. In 2014 the Centre received a total of 5,374 influenza samples from laboratories primarily in the Asia-Pacific region. Viruses were characterised by their antigenic, genetic and antiviral drug resistance properties. Of the viruses successfully analysed 52% were A(H1N1)pdm09 viruses. The majority of these were antigenically and genetically similar to the WHO recommended reference strain for the 2014 Southern Hemisphere influenza vaccine. Results for A(H3N2) and B/Yamagata viruses suggested that circulating viruses of this subtype and lineage, respectively, had undergone antigenic and/or genetic changes, consistent with the decision by WHO to change recommended strains for the 2015 Southern Hemisphere vaccine. A small number of A(H1N1)pdm09 and B/Victoria viruses had highly reduced inhibition to the neuraminidase inhibitors oseltamivir and zanamivir. The Centre also undertook primary isolation of vaccine candidate viruses directly into eggs. A total of 38 viruses were successfully isolated in eggs, of which 1 (B/Phuket/3073/2013) was included in the 2015 Southern Hemisphere influenza vaccine. *Commun Dis Intell* 2015;39(4):E584–E593.

### Introduction

The WHO Collaborating Centre for Reference and Research on Influenza in Melbourne (the Centre) is part of the World Health Organization's Global Influenza Surveillance and Response System (WHO GISRS). The GISRS network, established in 1952, monitors changes in influenza viruses with the aim of reducing the impact of influenza through the use of vaccines and antiviral medications. The Centre in Melbourne was first designated as a collaborating centre in 1992, the third such centre in the world. There are now 5 collaborating centres (Atlanta, Beijing, London, Melbourne and Tokyo) that analyse influenza viruses currently circulat-

ing in the human population. Virus samples are submitted to WHO collaborating centres by WHO National Influenza Centres and other hospital and regional laboratories. Based on data and advice from the 5 collaborating centres and other experts, the WHO makes biannual recommendations on suitable influenza strains to be included in the next seasonal vaccine (in February for the Northern Hemisphere and in September for the Southern Hemisphere). To this end, the Centre in Melbourne conducts a range of virological surveillance activities. This report summarises those surveillance activities undertaken in 2014.

### Virological surveillance performed by the Centre

Two types of influenza cause significant disease in humans: types A and B. Influenza A viruses are further classified into subtypes, based on their surface proteins, haemagglutinin (H) and neuraminidase (N). Thus, currently in circulation are subtypes A(H1N1)pdm09 and A(H3N2), although a number of subtypes have been known to infect humans and birds. Influenza B viruses are not classified into subtypes. However, there are currently two co-circulating lineages, B/Victoria/2/87 (B/Victoria) and B/Yamagata/16/88 (B/Yamagata). In addition, each year some cases of influenza C are isolated from humans, but as these viruses tend not to cause severe disease, they are not a focus of surveillance.

### Virus isolation

All virus isolates received at the Centre were re-passaged in cell culture (Madin-Darby Canine Kidney [MDCK] cells) and virus isolation was also attempted on a selection of original clinical specimens received. In addition, influenza-positive original clinical samples were directly inoculated into eggs as potential vaccine strains.

### Antigenic analysis

The antigenic properties of influenza viral isolates were analysed using the haemagglutination

inhibition (HI) assay as previously described.<sup>1</sup> In HI assays, viruses were tested for their ability to agglutinate red blood cells in the presence of ferret antisera previously raised against reference viruses. Isolates were identified as antigenically similar to the reference strain if the test samples had a titre that was no more than 4-fold different from the titre of the homologous reference strain. During 2014, results were reported by reference to the A/California/7/2009 (H1N1pdm09)-like, A/Victoria/361/2012 (H3N2)-like, B/Massachusetts/2/2012-like (Yamagata lineage), and B/Brisbane/60/2008-like (Victoria lineage) viruses that were recommended for the 2014 influenza vaccine.

### Genetic analysis

A subset of all influenza viruses analysed at the Centre underwent genetic analysis by sequencing the viral RNA genes. Viruses exhibiting evidence of antigenic variation or reduced antiviral drug susceptibility, as well as representative viruses from different time periods and geographic locations, were selected for sequencing of the haemagglutinin gene and/or the neuraminidase gene. Routine sequencing of the matrix protein for influenza A viruses and non-structural protein genes for influenza B viruses was also performed. In addition, the full genomes of a smaller subset of viruses were sequenced.

For sequencing, RNA was extracted from isolates or original clinical specimens using the QIAGEN Xtractor Gene robot, followed by reverse transcription polymerase chain reaction (RT-PCR) using the BIOLINE MYTAQ one step RT-PCR kit according to the manufacturer's recommendations with gene specific haemagglutinin primers (primer sequences available on request). Conventional Sanger sequencing was carried out on PCR product using an Applied Biosystems 3500 XL sequencer, and sequence assembly performed using the Seqman Pro Module of DNASTAR Lasergene version 9.1.0 software (DNASTar, Madison, WI, USA). Phylogenetic analysis was performed using MEGA 5.<sup>2</sup>

### Antiviral drug resistance testing

As the potential evolution of influenza viruses to develop resistance to antiviral drugs is of ongoing concern, circulating viruses were tested for their sensitivity to the currently used neuraminidase inhibitors oseltamivir (Tamiflu) and zanamivir (Relenza). The neuraminidase inhibition (NAI) assay used was a functional fluorescence-based assay in which the susceptibility of test viruses to the antiviral drugs was measured in terms of the drug concentration needed to reduce the neu-

raminidase enzymatic activity by 50% ( $IC_{50}$ ), and compared to values obtained with sensitive reference viruses of the same subtype or lineage. NAI assays were performed as previously described.<sup>3</sup> For the purposes of reporting, reduced inhibition of influenza A viruses was defined as a 10–99 fold increase in  $IC_{50}$ , while highly reduced inhibition was defined as a  $\geq 100$ -fold increase in  $IC_{50}$  in a NAI assay. For influenza B viruses, these figures were 5–49-fold and  $\geq 50$ -fold increases, respectively. However, it should be noted that the relationship between the  $IC_{50}$  value and the clinical effectiveness of a neuraminidase inhibitor is not well understood and reduced inhibition may not be clinically significant.

Viruses found to have highly reduced inhibition by either oseltamivir or zanamivir underwent further analysis to determine the presence of amino acid substitutions in the neuraminidase protein that were associated with the reduction of inhibition by NAI assays. For example, a change from histidine to tyrosine at position 275 (H275Y) of the neuraminidase protein of A(H1N1)pdm09 viruses is known to reduce inhibition by oseltamivir, as does the H273Y NA mutation in influenza B viruses.<sup>4</sup>

### Candidate vaccine strains

The viruses used to produce human vaccines are required to be isolated and passaged in embryonated hen's eggs or certified cell lines. The Centre undertook primary isolation of selected viruses from clinical samples directly into eggs, using previously described methods.<sup>5</sup> These isolates were then analysed by HI assay and genetic sequencing.

## Results

During 2014, a total of 5,374 clinical specimens and/or virus isolates were received by the Centre from 36 laboratories in 14 countries (Figure 1). Most of the samples were provided by laboratories in the Asia–Pacific region. Figure 2 shows the temporal distribution of samples sent to the Centre by subtype and lineage. Most samples were received during the Australian influenza season.

### Influenza A(H1N1)pdm09

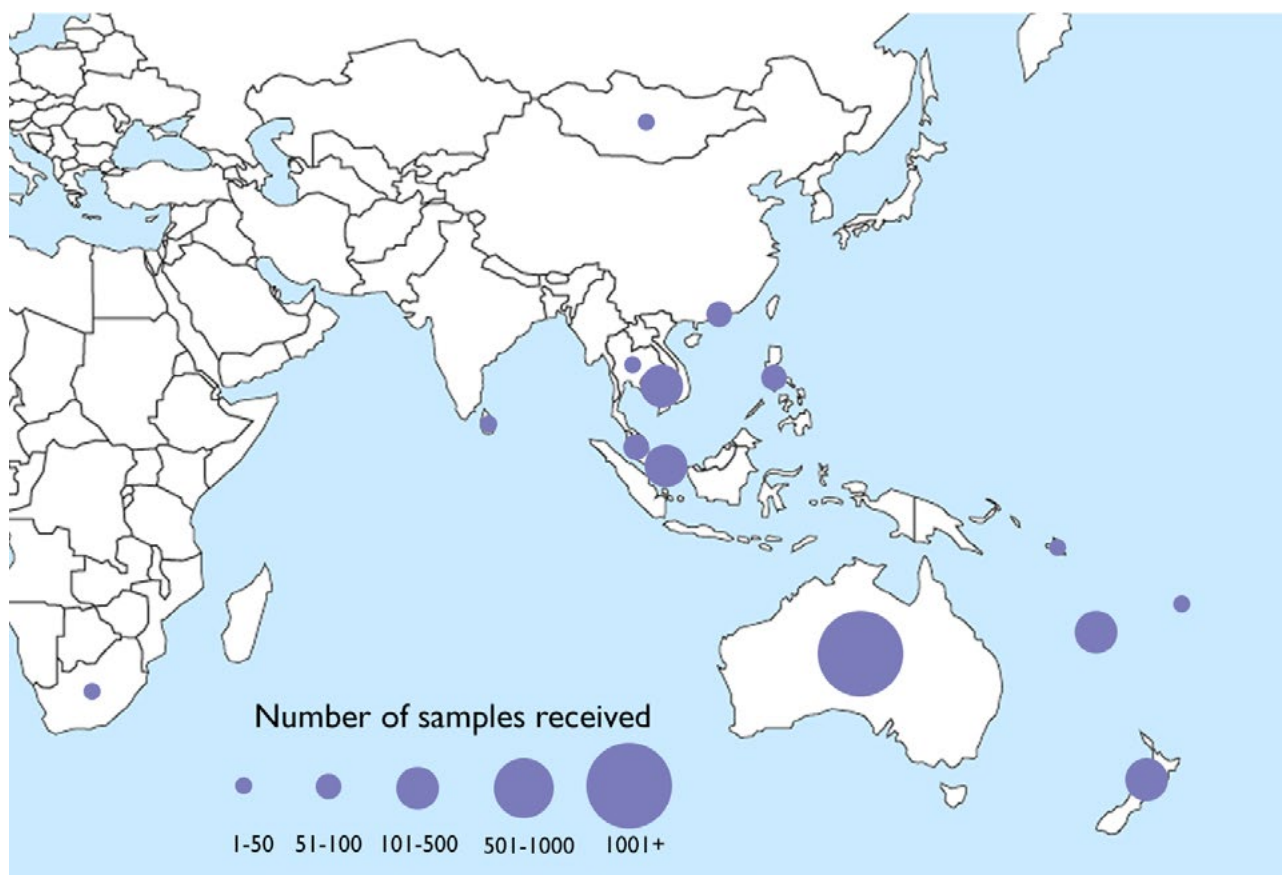
Virus isolation was attempted for 4,899 (91%) of the samples received and was successful in 3,382 cases (69%). Of these, 3,374 were characterised by HI assay (Table). In addition, 27 samples were characterised by RT-PCR. Some 444 haemagglutinin genes were sequenced. Full genome sequencing using Sanger sequencing techniques was performed on 63 viruses. Of the samples for which results could be obtained ( $n=3,404$ ), 52%

were identified as A(H1)pdm09, 29% were A(H3) viruses, 17% were B/Yamagata, 2% were B/Victoria and there was 1 sample with mixed H3/B viruses.

Of the 1,761 A(H1)pdm09 isolates analysed by HI assay in 2014, the majority (99.5%) were antigenically similar to the vaccine reference strain A/California/7/2009 (Table).

Sequencing and phylogenetic analysis of haemagglutinin genes from 168 viruses showed that A(H1N1)pdm09 viruses sent to the Centre during 2014 contained some minor genetic changes compared with A/California/7/2009 (Figure 3). However, the antigenic behaviour of these viruses was not affected by these changes.

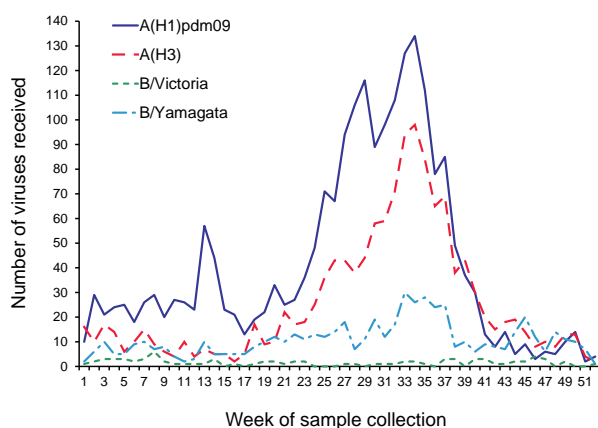
**Figure 1: Geographic spread of influenza laboratories sending viruses to the Centre during 2014**



**Table: Antigenic analysis of viruses received by the Centre, by country of origin**

Region	A(H1)pdm09: A/California/7/2009 (cell)		A(H3) : A/Texas/50/2012 (cell)		B/Victoria: B/Brisbane/60/2008 (cell)		B/Yamagata: B/Massachusetts/2/2012 (cell)	
	Like	Low reactor (%)	Like	Low reactor (%)	Like	Low reactor (%)	Like	Low reactor (%)
Australasia	1,492	7 (0.5%)	647	41 (5.9%)	32	0	131	243 (65%)
Pacific	54	0	185	5 (2.6%)	2	0	3	6 (67%)
South East Asia	175	1 (0.6%)	30	17 (3.6%)	29	1 (3.3%)	98	66 (40%)
East Asia	25	1 (3.8%)	18	2 (10%)	16	0	5	12 (71%)
South Asia	2	0	8	0	0	0	0	0 (0%)
Africa	4	0	11	0	0	0	1	2 (67%)
Total	1,752	9 (0.5%)	899	65 (6.7%)	79	1 (1.3%)	238	329 (58%)

**Figure 2: Number of samples received at the Centre, 2014, by week of sample collection**



Nineteen viruses were inoculated into eggs for vaccine candidate strain isolation. Of these, 10 (53%) were successfully isolated and included at least 1 virus from each of the 3 clades represented in the dendrogram (Figure 3).

Of 1,743 H1 viruses tested, seven exhibited highly reduced inhibition by oseltamivir. These viruses were from Australia (Perth,  $n=3$ ), Macau, Malaysia, Thailand, and New Caledonia. All of these viruses had the H275Y mutation. No H1 viruses showed highly reduced inhibition by zanamivir.

### Influenza A(H3N2)

Antigenic analysis of 964 A(H3) subtype isolates showed that only 7% were low reactors to the cell-propagated reference strain A/Texas/50/2012 (Table). However, 24% of viruses were low reactors to the egg-propagated strain A/Texas/50/2012.

A total of 155 haemagglutinin genes from A(H3N2) viruses were sequenced. Phylogenetic analysis indicated that recently circulating viruses had undergone significant genetic changes compared with A/Texas/50/2012 (Figure 4). These viruses fell into one of 3 clades, designated 3C.2a and 3C.3a and 3C.3b, while A/Texas/50/2012 was in clade 3C.1.

Seventy-eight viruses were inoculated into eggs, of which 23 (29%) grew successfully. These viruses were representative of each of the clades identified in Figure 4 and included at least 4 viruses from clade 3C.2a and 2 viruses from clade 3C.3a.

None of the 969 H3 viruses tested had highly reduced inhibition by either of the neuraminidase inhibitors.

### Influenza B

There are currently 2 antigenically and genetically distinct lineages of influenza B virus in circulation, the B/Victoria/2/87 lineage (represented by B/Brisbane/60/2008) and the B/Yamagata/16/88 lineage (represented by the southern hemisphere 2014 vaccine strain B/Massachusetts/2/2012). Among influenza B viruses received at the Centre during 2014, the B/Yamagata lineage was predominant (Table).

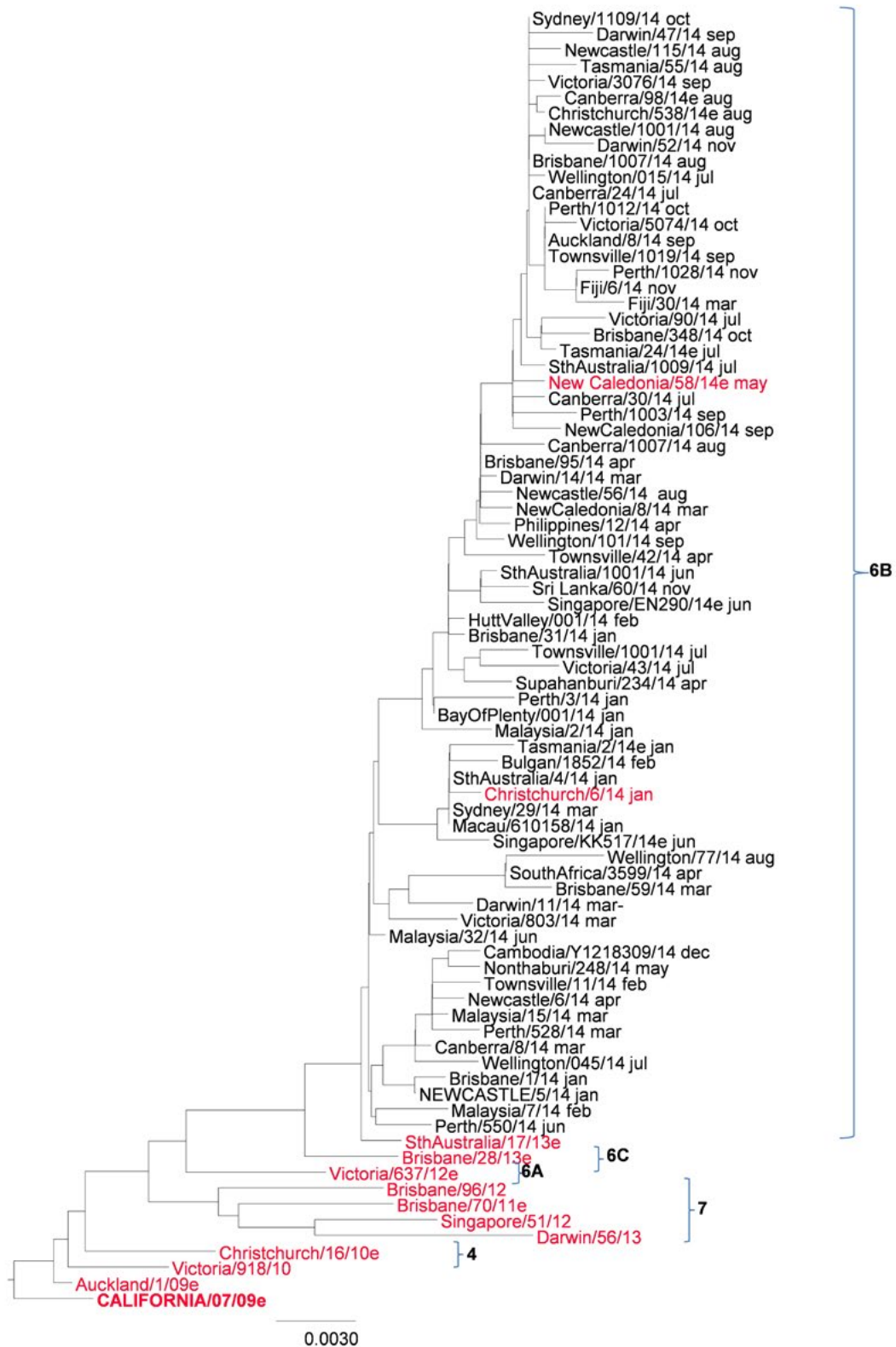
A total of 647 influenza B viruses were characterised by HI assay. All but one of the 80 B/Victoria viruses received and analysed were similar to B/Brisbane/60/2008 (Table). The majority (58%) of the 567 B/Yamagata lineage viruses analysed were low reactors to the B component of the 2014 vaccine, B/Massachusetts/2/2012 (Table). In contrast, roughly 66% were antigenically similar to the 2013 vaccine strain, B/Wisconsin/1/2010.

Sequencing was performed on 121 haemagglutinin genes from B viruses, the majority being B/Yamagata viruses. All of the viruses of B/Victoria lineage were genetically similar to the B/Brisbane/60/2008 reference virus (Figure 5). The majority of B/Yamagata lineage viruses belonged to Clade 3 and were antigenically and genetically distinct from the 2014 vaccine strain B/Massachusetts/2/2012 (Clade 2; Figure 6). Clade 3 includes B/Phuket/3073/2013, which is the B strain included in the 2015 vaccine, as well as B/Wisconsin/1/2010, which was the B strain used in the 2013 influenza vaccine.

Egg isolation was attempted for 7 B/Victoria and 16 B/Yamagata viruses. None of the B/Victoria viruses were successfully isolated, while 31% of the B/Yamagata viruses were isolated in eggs. One of these was the B/Phuket/3073/2013, the virus that was included in the 2015 Southern Hemisphere influenza vaccine.

Of 80 B/Victoria viruses tested, one from Mongolia displayed highly reduced inhibition by both oseltamivir and zanamivir. This virus had the G104R mutation in the NA gene. None of the 566 B/Yamagata viruses tested showed highly reduced inhibition by the neuraminidase inhibitors.

Figure 3: Phylogenetic tree of representative haemagglutinin genes of A(H1N1)pdm09 viruses, 2014



2015 Southern Hemisphere vaccine strain is presented in capital letters

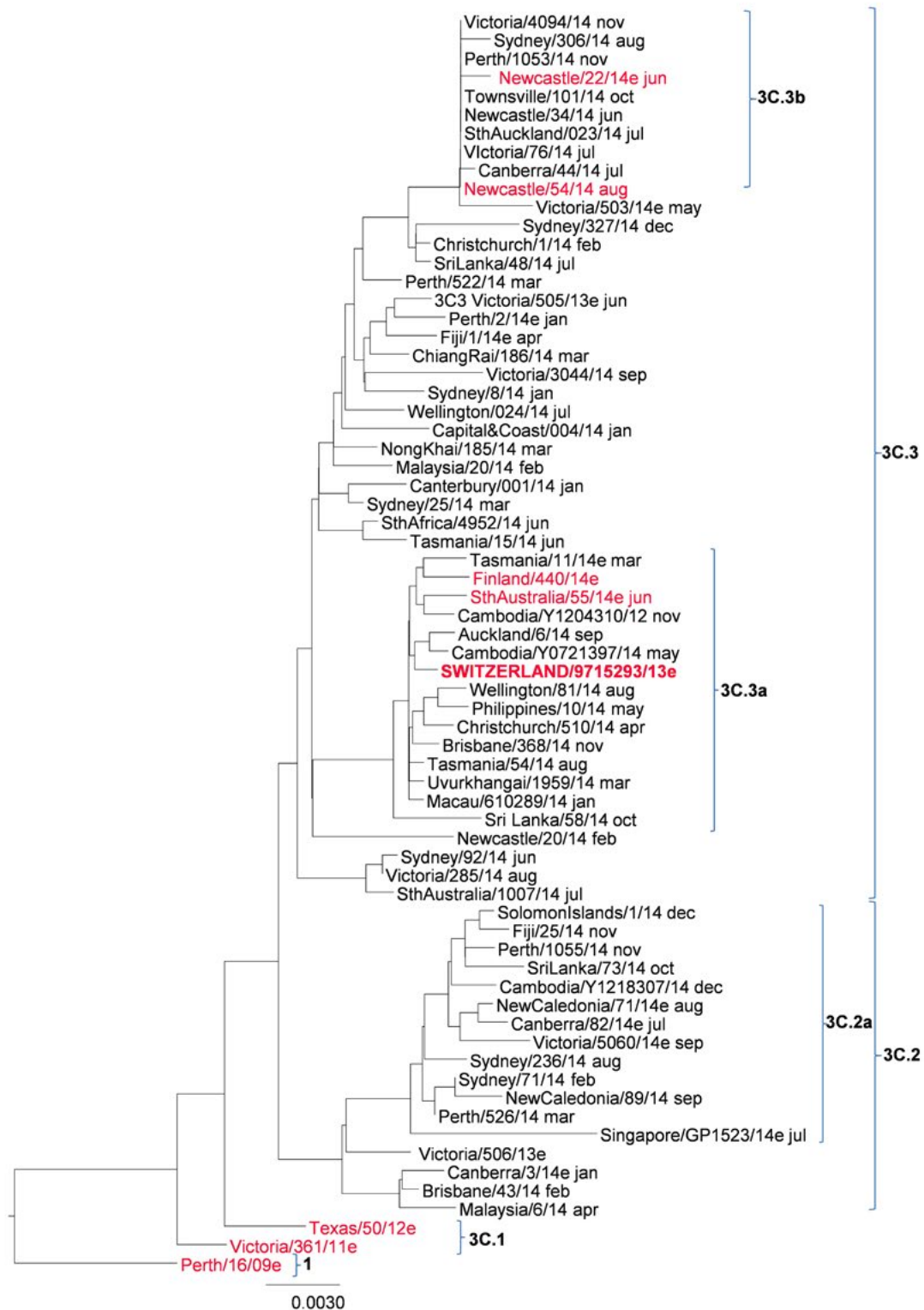
Reference virus is indicated in red text

e: egg isolate

Scale bar represents 0.3% nucleotide sequence difference between viruses

} Braces indicate clades

Figure 4. Phylogenetic tree of representative haemagglutinin genes of A(H3N2) viruses, 2014



2015 Southern Hemisphere vaccine strain is presented in capital letters

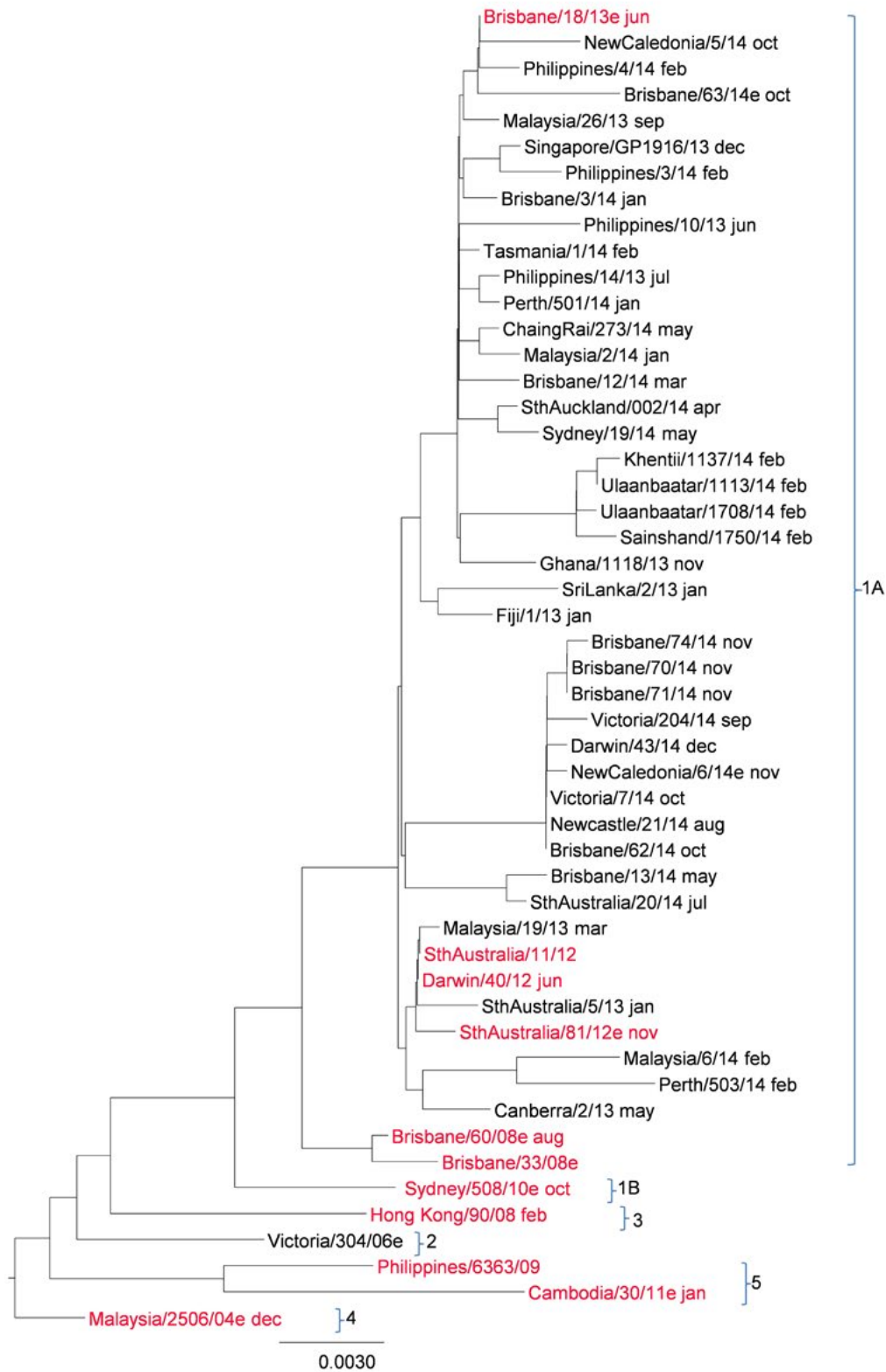
Reference virus is indicated in red text

e: egg isolate

Scale bar represents 0.3% nucleotide sequence difference between viruses

} Braces indicate clades

Figure 5. Phylogenetic tree of representative haemagglutinin genes of B/Victoria viruses, 2014



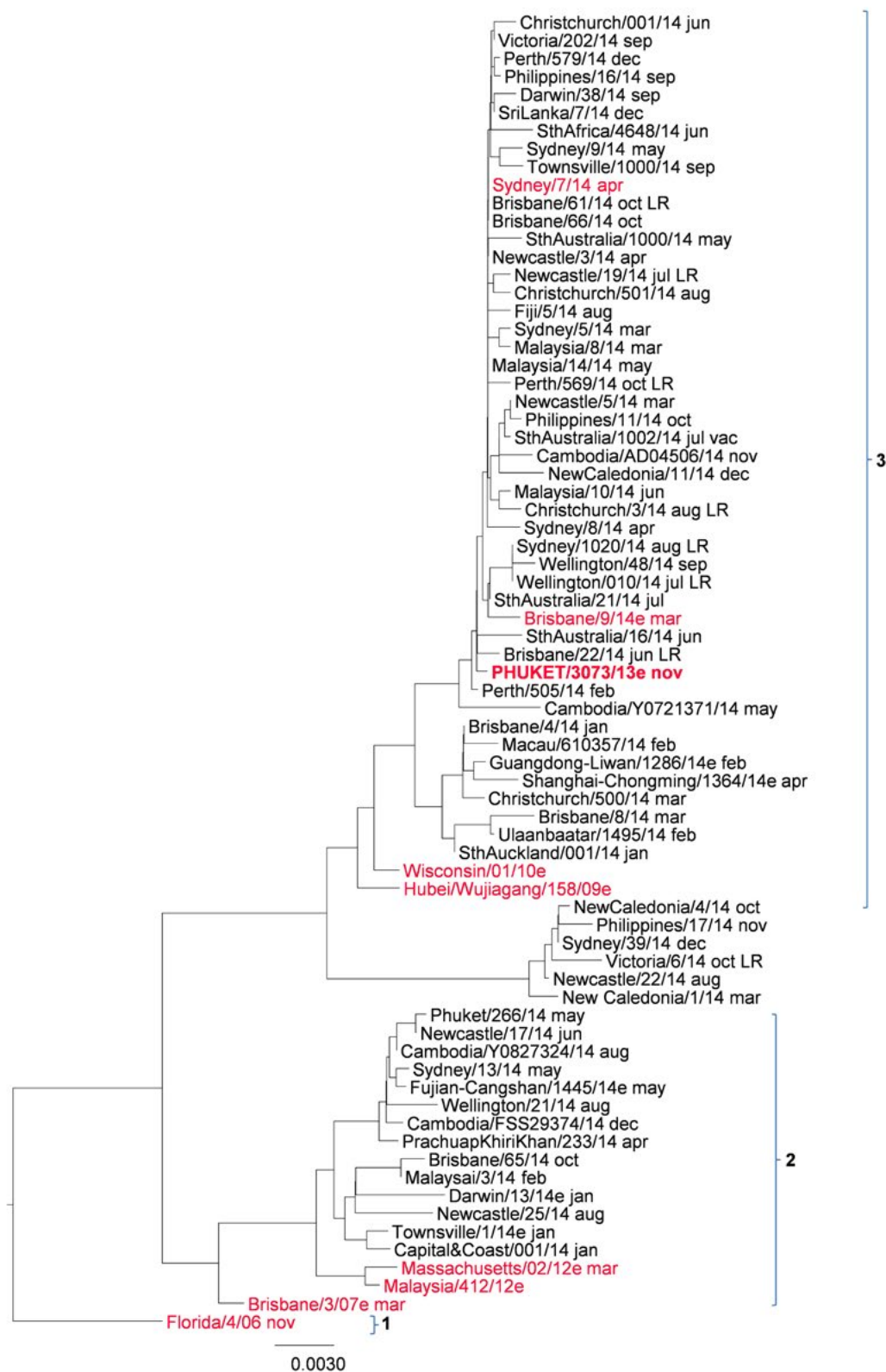
Reference virus is indicated in red text

e: egg isolate

Scale bar represents 0.3% nucleotide sequence difference between viruses

} Braces indicate clades

Figure 6. Phylogenetic tree of representative haemagglutinin genes of B/Yamagata viruses, 2014



2015 Southern Hemisphere vaccine strain is presented in capital letters

Reference virus is indicated in red text

e: egg isolate

Scale bar represents 0.3% nucleotide sequence difference between viruses

} Braces indicate clades

## Discussion

The viruses analysed by the Centre were heavily dominated by samples received from Australian laboratories. The National Notifiable Diseases Surveillance System (NNDSS) data indicated that in 2014 Australia had the highest influenza activity on record, with 67,709 notifications of laboratory confirmed influenza.<sup>6</sup> This was reflected in the large number of samples received at the Centre, second only to 2009, the year of the A(H1N1)pdm09 pandemic, when 6,435 samples were received.

National Influenza Centre data indicated that A(H1)pdm09 viruses predominated in Australia in 2014.<sup>7</sup> This was reflected in the samples received by the Centre, where the majority of viruses received were A(H1)pdm09. Antigenic and genetic data indicated a good match between the vaccine strain and the circulating strains. Indeed, interim estimates from New Zealand, which also used the WHO-recommended Southern Hemisphere vaccine composition, observed that the vaccine's effectiveness was good (73% [95%CL: 50,85]).<sup>8</sup>

Despite the overall predominance of A(H1N1)pdm09 viruses during 2014, influenza A(H3N2) predominated in New South Wales and the Australian Capital Territory, and towards the end of the year an increasing number of A(H3N2) viruses from Queensland, Western Australia, the Northern Territory and Tasmania were reported to NNDSS.<sup>7</sup> As a result, the proportion of H3 viruses received at the Centre among all viruses increased towards the end of the 2014 season.

HI assays performed at the Centre showed that at least 75% of H3 viruses tested were antigenically similar to the egg-grown A/Texas/50/2011 strain. However, recently there have been considerable challenges in the antigenic characterisation of H3 viruses. Evolutionary changes in this subtype have made it difficult to detect antigenic changes using the HI assay.<sup>9</sup> Although other assays (e.g. virus neutralisation) are being used to examine the antigenicity of H3 viruses, these methods are more labour intensive and cannot easily replace the HI assay. Studies are continuing to determine the most appropriate method for detecting antigenic changes in recent H3 viruses, but more emphasis is being placed on genetic analysis. Genetic data from the Centre indicated that many 2014 H3 viruses fell in clades 3C.2a, 3C.3a and 3C.3b (the 2015 Southern Hemisphere vaccine strain, A/Switzerland/9715293/2013, lies in clade 3C.3a). The clades appear to be antigenically distinct. Reports in early 2015 from several Northern Hemisphere countries suggested low effectiveness against A(H3N2) for the vaccines containing A/

Texas/50/2012.<sup>10–13</sup> This has been attributed to increased circulation of clade 3C.2a viruses, while the Texas strain is a clade 3C.1 virus.<sup>10</sup>

A further challenge in the development of H3 vaccine candidates has arisen in recent years. When isolated in eggs, human A(H3N2) viruses rapidly acquire adaptive changes in the haemagglutinin, which may alter antigenicity. This has been a problem for a long time but was a particular issue with the A/Victoria/361/2011 vaccine virus included in the 2013 vaccine.<sup>14</sup> This has stimulated a major effort to obtain H3 egg isolates that retain the antigenic properties of their corresponding cell isolates.

Finally, a majority of influenza B/Yamagata-lineage viruses received by the Centre appeared to be antigenically distant from the vaccine virus, B/Massachusetts/2/2012. This virus was selected for the vaccine in light of evidence that B/Massachusetts/2/2012-like viruses were increasingly prevalent in 2013. However, viruses that were antigenically more similar to the 2013 vaccine strain B/Wisconsin/1/2010 unexpectedly dominated in 2014. These 2 strains are both B/Yamagata viruses, but sit within different clades in the phylogenetic tree. There are no published vaccine effectiveness estimates available for influenza B during the 2014 or 2014/15 seasons. Genetic analysis by the Centre showed that many of the viruses were genetically similar to the egg-propagated B/Phuket/3073/2013 virus isolated at the Centre and this virus was subsequently recommended for inclusion in the Southern Hemisphere 2015 vaccine.

## Author details

Dr Sheena G Sullivan  
Dr Michelle K Chow  
Dr Ian G Barr  
Professor Anne Kelso

The Melbourne WHO Collaborating Centre for Reference and Research on Influenza is supported by the Australian Government Department of Health.

Corresponding author: Dr Sheena Sullivan, WHO Collaborating Centre for Reference and Research on Influenza, Peter Doherty Institute for Infection and Immunity, 792 Elizabeth Street, MELBOURNE VIC 3000. Telephone: +61 3 9342 9317. Email: [sheena.sullivan@influenzacentre.org](mailto:sheena.sullivan@influenzacentre.org)

## References

1. Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. *J Hyg (Lond)* 1972;70(4):767–777.
2. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S. MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods. *Mol Biol Evol* 2011;28(10):2731–2739.

3. Hurt AC, Okomo-Adhiambo M, Gubareva LV. The fluorescence neuraminidase inhibition assay: a functional method for detection of influenza virus resistance to the neuraminidase inhibitors. *Methods Mol Biol* 2012;865:115–125.
4. Takashita E, Meijer A, Lackenby A, Gubareva L, Rebelo-de-Andrade H, Besselaar T, et al. Global update on the susceptibility of human influenza viruses to neuraminidase inhibitors, 2013–2014. *Antiviral Res* 2015;117:27–38.
5. WHO Global Influenza Surveillance Network. *Manual for the laboratory diagnosis and virological surveillance of influenza*. Geneva: World Health Organization; 2011.
6. Australian Government Department of Health. National Notifiable Diseases Surveillance System. 2015. Accessed on 6 May 2015. Available from: <http://www9.health.gov.au/cda/source/cda-index.cfm>
7. Australian Government Department of Health. Australian Influenza Surveillance Report. No. 8, 2014, 27 September to 10 October 2014; 2014.
8. Turner N, Pierse N, Huang QS, Radke S, Bissielo A, Thompson MG, et al. Interim estimates of the effectiveness of seasonal trivalent inactivated influenza vaccine in preventing influenza hospitalisations and primary care visits in Auckland, New Zealand, in 2014. *Euro Surveill* 2014;19(42) pii: 20934.
9. Barr IG, Russell C, Besselaar TG, Cox NJ, Daniels RS, Donis R, et al. WHO recommendations for the viruses used in the 2013–2014 Northern Hemisphere influenza vaccine: Epidemiology, antigenic and genetic characteristics of influenza A(H1N1)pdm09, A(H3N2) and B influenza viruses collected from October 2012 to January 2013. *Vaccine* 2014;32(37):4713–4725.
10. Skowronski DM, Chambers C, Sabaiduc S, De Serres G, Dickinson JA, Winter AL, et al. Interim estimates of 2014/15 vaccine effectiveness against influenza A(H3N2) from Canada's Sentinel Physician Surveillance Network, January 2015. *Euro Surveill* 2015;20(4): pii: 21022.
11. Pebody R, Warburton F, Ellis J, Andrews N, Thompson C, von Wissmann B, et al. Low effectiveness of seasonal influenza vaccine in preventing laboratory-confirmed influenza in primary care in the United Kingdom: 2014/15 mid-season results. *Euro Surveill* 2015;20(5):21025.
12. McNeil SA, Andrew MK, Ye L, Haguinet F, Hachette TF, ElSherif M, et al. Interim estimates of 2014/15 influenza vaccine effectiveness in preventing laboratory-confirmed influenza-related hospitalisation from the Serious Outcomes Surveillance Network of the Canadian Immunization Research Network, January 2015. *Euro Surveill* 2015;20(5):21024.
13. Flannery B, Clippard J, Zimmerman RK, Nowalk MP, Jackson ML, Jackson LA, et al. Early estimates of seasonal influenza vaccine effectiveness—United States, January 2015. *MMWR Morb Mortal Wkly Rep* 2015;64(1):10–15.
14. Skowronski DM, Janjua NZ, De Serres G, Sabaiduc S, Eshaghi A, Dickinson JA, et al. Low 2012–13 influenza vaccine effectiveness associated with mutation in the egg-adapted H3N2 vaccine strain not antigenic drift in circulating viruses. *PLoS One* 2014;9(3):e92153.