Research article

Mutations acquired during cell culture isolation may affect antigenic characterisation of influenza A(H3N2) clade 3C.2a viruses

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As elsewhere, few (<15%) sentinel influenza A(H3N2) clade 3C.2a viruses that dominated in Canada during the 2014/15 season could be antigenically characterised by haemagglutination inhibition (HI) assay. Clade 3C.2a viruses that could be HI-characterised had acquired genetic mutations during in vitro cell culture isolation that modified the potential glycosylation motif found in original patient specimens and the consensus sequence of circulating viruses at amino acid positions 158–160 of the haemagglutinin protein. Caution is warranted in extrapolating antigenic relatedness based on limited HI findings for clade 3C.2a viruses that continue to circulate globally.

Introduction

During the 2014/15 influenza A(H3N2) epidemic, viruses belonging to phylogenetic clade 3C.2a predominated [1,2]. Viruses within this clade bore multiple (10–12) amino acid differences from the A/Texas/50/2012 (clade 3C.1) vaccine strain at antigenic sites of the surface haemagglutinin (HA) protein [2]. These differences included two clade-defining substitutions, a phenylalanine (F) to tyrosine (Y) substitution at residue 159 (F159Y) and a lysine (K) to threonine (T) substitution at the adjacent residue 160 (K160T), both in antigenic site B [1,2], a highly exposed region at the top of the HA protein where mutations create the potential for viral evasion of the antibody response [3,4]. Together with an asparagine (N) residue at position 158, conserved in all clade 3C viruses, the N158-Y159-T160 sequon in clade 3C.2a viruses represents a potential gain of glycosylation that can mask viral epitopes and reduce antibody access to the immuno-dominant antigenic site B [5,6]. This potential glycosylation motif at amino acid positions 158–160 of the HA protein is unique to clade 3C.2a viruses, and is not found in other recently circulating A(H3N2) genetic clades.

Koel et al. have previously highlighted positions 158 and 159 as among seven residues in the HA protein associated with all major antigenic cluster-transition events in A(H3N2) viruses since 1968 [7] and in a recent serological analysis, Chambers et al. highlighted the substitution at position 159 as likely to have been responsible for the 2014/15 antigenic drift [8]. Consistent with these molecular findings, mid-season vaccine effectiveness (VE) analyses from multiple countries, including the Canadian Sentinel Practitioner Surveillance Network (SPSN), the United States (US) and the United Kingdom (UK), reported negligible protection against the 2014/15 A(H3N2) clade 3C.2a epidemic strain [2,9,10]. In February 2015, the World Health Organization (WHO) recommended that the A(H3N2) component for the 2015/16 season be updated to an A/Switzerland/9715293/2013-like (clade 3C.3a) strain [11].

Influenza surveillance reports from reference laboratories globally have indicated that circulating A(H3N2) viruses belonging to clade 3C.2a are antigenically

Table 1

Haemagglutination inhibition assay titres and fold reductions relative to cell- and egg-passaged A/Switzerland/9715293/2013 reference virus for sentinel A(H3N2) virus isolates with known genetic clade, Canadian Sentinel Practitioner Surveillance Network, November 2014–April 2015 (n = 49)

Sentinel isolate (clade)	Cell-passaged A/Switzerland/9715293/2013 (n = 49)				Egg-passaged A/Switzerland/9715293/2013 (n = 35[a])			
	n	Sentinel isolate HI titre range	Homologous reference virus HI titre range	Fold reduction	n	Sentinel isolate HI titre range	Homologous reference virus HI titre range	Fold reduction
Clade 3C.2a	31	80–160	320–640	≤ 4	25	160–320	320–640	≤ 4
Clade 3C.3	1	160	320	2	1	320	640	2
Clade 3C.3a	2	160–640	320–640	≤ 4	2	320	320–640	≤ 2
Clade 3C.3b	15	80–1,280	320	≤ 4	7	80–640	320–640	≤ 8[b]

HI: haemagglutination inhibition.

[a] These 35/49 viruses were characterised in relation to both the cell-passaged and egg-passaged A/Switzerland/9715293/2013 reference virus. For the other 14/49 viruses initially characterised in relation to the cell-passaged A/Switzerland/9715293/2013 reference virus, there remained insufficient viral titre to support further characterisation in relation to the egg-passaged reference virus.

[b] One clade 3C.3b virus had an eightfold reduction to egg-passaged A/Switzerland/9715293/2013 reference virus; all other tested viruses had ≤ 4-fold reductions.

Table 2

Potential glycosylation motif at haemagglutinin positions 158-160 in influenza A(H3N2) clade 3C.2a viruses from original patient specimens and culture isolates prior to haemagglutination inhibition assay by the NML, Canadian Sentinel Practitioner Surveillance Network, November 2014–April 2015 (n = 234)

HI assay characterisation	HA amino acid sequence at positions 158-159-160					
	Original patient specimens n = 234			Culture isolates n = 234		
	N-Y-T[a] n = 219	Not N-Y-T n = 2	Sequence not available n = 13	N-Y-T[a] n = 156	Not or poly N-Y-T n = 71	Sequence not available n = 7
Sufficient HA titre for HI assay[b] (n = 31)	26 (12)	1	4	0 (0)	28 (39)	3
Insufficient HA titre for HI assay[b] (n = 203)	193 (88)	1	9	156 (100)	43 (61)	4

HA: haemagglutinin; HI: haemagglutination inhibition; NML: National Microbiology Laboratory; poly: polymorphic for the N-Y-T amino acid sequence (i.e. partial loss of the potential glycosylation motif).

Values displayed are: number (% column).

[a] A potential glycosylation motif is defined by the amino acid sequon: N-X-T/S; where N is asparagine, X is any amino acid other than proline and T/S is either threonine or serine [5,6]. The consensus sequence for clade 3C.2a viruses is N158-Y159-T160, conferring a potential gain of glycosylation.

[b] In the presence of 20 nM oseltamivir.

related to A/Switzerland/9715293/2013, despite the fact that only a small proportion could be characterised by conventional haemagglutination inhibition (HI) assay [1,12,13]. Influenza A(H3N2) viruses have been difficult to characterise antigenically by HI assay due to variable agglutination of erythrocytes or loss of ability to agglutinate erythrocytes, a particular problem for clade 3C.2a viruses [1]. For the majority of A(H3N2) viruses that could not be HI-characterised, reference laboratories have imputed antigenic relatedness based on sequencing findings, assuming that viruses that could be characterised within a given genetic group or clade are broadly representative of circulating strains [12,13].

The European Centre for Disease Prevention and Control (ECDC) has earlier highlighted that clade 3C.2a viruses that had sufficient HA titre to agglutinate erythrocytes and that could be characterised by HI assay had either lost or were polymorphic for the clade-defining glycosylation motif at positions 158–160 [14]. To assess the representativeness of clade 3C.2a viruses that could be characterised by HI assay, we examined amino acid identity at positions 158–160 for the presence of this potential glycosylation motif in original patient specimens collected by the Canadian SPSN compared with the corresponding sequence after cell culture isolation of virus during the 2014/15 season.

Methods

Nasal/nasopharyngeal specimens collected from patients within seven days of influenza-like illness onset through the Canadian SPSN between 1 November 2014 and 30 April 2015 were tested for influenza by RT-PCR. Influenza-positive specimens were inoculated into Madin-Darby Canine Kidney (MDCK), MDCK-SIAT1 or Rhesus Monkey Kidney (RMK) cells to attempt culture isolation as per provincial reference laboratory protocols. Cell culture isolates were submitted to Canada's National Microbiology Laboratory (NML) for antigenic characterisation by standard HI assay protocols using guinea pig erythrocytes and post-infection ferret antisera supplied by the US Centers for Disease Control and Prevention (US CDC) raised against cell- and egg-passaged A/Switzerland/9715293/2013 reference viruses [2]. To circumvent any neuraminidase (NA)-mediated binding of A(H3N2) viruses to erythrocytes, HI assays were conducted in the presence of 20 nM oseltamivir carboxylate following, where indicated, a further single passage in MDCK-SIAT1 cells at the NML to improve viral titres [15,16]. Antigenic relatedness of a sentinel isolate to A/Switzerland/9715293/2013 reference virus was defined as a ≤4-fold reduction in HI antibody titre compared to the titre of the homologous reference virus [17].

Sanger sequencing of the viral HA gene was conducted on the original patient specimens to establish clade designation and to detect amino acid substitutions in HA antigenic sites. For the current study, sequencing was also conducted on cultured isolates of clade 3C.2a viruses before and after further passage in MDCK-SIAT1 cells (if indicated) prior to HI characterisation at the NML to assess amino acid identity relative to the clade 3C.2a N158-Y159-T160 consensus sequence and to sequences based on the corresponding original patient specimen. Ethics boards in each participating province approved the SPSN VE study of which this virological sub-analysis is a component; virus characterisation was also conducted as part of national surveillance activities.

Results

Clade distribution and HI characterisation

Of the 460 influenza A(H3N2) detections by the SPSN during the 2014/15 season with known clade information, 265 (58%) virus isolates were cultivated by provincial reference laboratories and submitted to the NML for antigenic characterisation by HI assay. Of the 265 virus isolates, 197 (74%) were grown by provincial laboratories in MDCK, 44 (17%) in MDCK-SIAT1 and 24 (9%) in RMK cells. Submitted A(H3N2) virus isolates included 234 (88%) viruses belonging to clade 3C.2a, 25 (9%) belonging to clade 3C.3b, four (2%) belonging to clade 3C.3 and two (1%) belonging to clade 3C.3a, reflecting the overall clade distribution and clade 3C.2a predominance among sentinel A(H3N2) detections previously reported [2].

Of these 265 virus isolates with known clade information, 49 (18%) had sufficient HA titre to agglutinate erythrocytes and could be characterised by HI assay. These included only 31 (13%) of the 234 virus isolates belonging to clade 3C.2a. By comparison, of the 31 non-clade 3C.2a virus isolates, 18 could be characterised by HI, including 15 of 25 belonging to clade 3C.3b, one of four belonging to 3C.3, and both of the virus isolates belonging to clade 3C.3a.

All 49 virus isolates that could be HI-characterised were considered antigenically related to the cell-passaged A/Switzerland/9715293/2013 vaccine prototype recommended for the 2015/16 vaccine. A subset of 35 of the 49 viruses was additionally characterised against the egg-passaged A/Switzerland/9715293/2013 vaccine reference and 34 of them were considered antigenically related; one clade 3C.3b virus showing eightfold titre reduction was considered antigenically distinct (**Table 1**).

Clade 3C.2a viruses and the 158–160 sequon

Of the 234 clade 3C.2a virus isolates submitted to NML, sequencing of the viral HA at positions 158–160 based on original patient specimens was successful for 221 (94%) viruses (**Table 2**). Of these 221 viruses from original patient specimens, 219 (99%) bore the clade 3C.2a consensus sequence N158-Y159-T160 consistent with the potential glycosylation motif and two (1%) instead bore K160 found otherwise in clade 3C.2 viruses.

Following cell culture isolation at provincial reference laboratories, 229 of 234 (98%) clade 3C.2a virus isolates had sequencing information available before MDCK-SIAT1 passage (if indicated) at the NML. Of these, 63 (28%) viruses had lost or partially lost (i.e. become polymorphic for) the N158-Y159-T160 consensus sequence, including 45 (25%) of 178 grown in MDCK, 10 of 30 grown in MDCK-SIAT1 and eight of 21 grown in RMK cells.

Of the 31 clade 3C.2a viruses that could be HI-characterised, 17 had undergone a single further passage in MDCK-SIAT1 cells at the NML prior to HI assay. Sequencing information was available for 15 of these 17 viruses that required additional MDCK-SIAT1 passage and 13 of the 14 viruses that did not require further MDCK-SIAT1 passage. Based on available sequencing information, all 28 viruses had lost or were polymorphic for the potential glycosylation motif (**Table 2**). For all but two of the virus isolates that were further passaged in MDCK-SIAT1 cells at the NML, sequences were identical before and after that passage. Of the two viruses modified with MDCK-SIAT1 passage at the NML, one isolate that was polymorphic after initial cell culture lost the potential glycosylation motif and one isolate that maintained the original consensus sequence after initial cell culture became polymorphic after MDCK-SIAT1 passage.

Table 3

Amino acid sequence at haemagglutinin positions 158–160 of influenza A(H3N2) clade 3C.2a viruses with respect to the potential glycosylation motif in final culture isolates prior to haemagglutination inhibition assay by the NML, Canadian Sentinel Practitioner Surveillance Network, November 2014–April 2015 (n = 234)

HA amino acid sequence			Glycosylation motif	Frequency n (%)	Interpretation
158	159	160			
Consensus sequence in circulating clade 3C.2a viruses					
N	Y	T	+CHO		
Sequence after final cell culture[a]					
A(H3N2) clade 3C.2a viruses with sufficient HA titre for HI assay[b] (n = 31)					
N	Y	K	– CHO	8	Reversion to clade 3C.2 K160
N	Y	A	– CHO	7	New T160A mutation
N	Y	T/K	Polymorphic[c]	4	Partial reversion to clade 3C.2 K160
K	Y	T	– CHO	3	New N158K mutation
N	Y	I	– CHO	3	New T160I mutation
D	Y	T	– CHO[d]	1	New N158D mutation
H	Y	T	– CHO	1	New N158H mutation
N/S	Y	T	Polymorphic	1	Polymorphic for new N158S mutation
Sequence not available			NA	3	NA
A(H3N2) clade 3C.2a viruses with insufficient HA titre for HI assay[b] (n = 203)					
N	Y	T	+CHO	156 (77)	Consensus clade 3C.2a sequence
N/K	Y	T	Polymorphic	6 (3)	Polymorphic for new N158K mutation
N/D	Y	T	Polymorphic	5 (2)	Polymorphic for new N158D mutation
N	Y	T/I	Polymorphic	5 (2)	Polymorphic for new T160I mutation
N	Y	T/A	Polymorphic	5 (2)	Polymorphic for new T160A mutation
N	Y	T/K	Polymorphic	5 (2)	Partial reversion to clade 3C.2 K160
N	Y	A	– CHO	3 (1)	New T160A mutation
N	Y	I	– CHO	3 (1)	New T160I mutation
N	Y	K	– CHO	3 (1)	Reversion to clade 3C.2 K160
S	Y	T	– CHO	2 (1)	New N158S mutation
D	Y	T	– CHO	1 (0)	New N158D mutation
K	Y	T	– CHO	1 (0)	New N158K mutation
N/K	Y	T/I	Polymorphic	1 (0)	Polymorphic for new N158K and T160I mutations
N/R	Y	T/I	Polymorphic	1 (0)	Polymorphic for new N158R and T160I mutations
N/S	Y	T/A	Polymorphic	1 (0)	Polymorphic for new N158S and T160A mutations
N/K/R/S	Y	T	Polymorphic	1 (0)	Polymorphic for new N158K/R/S mutation
Sequence not available			NA	4 (2)	NA

CHO: carbon-hydrogen-oxygen (i.e. glycosylation); HA: haemagglutinin; HI: haemagglutination inhibition; NA: not available; NML: National Microbiology Laboratory.

+CHO: potential glycosylation motif in clade 3C.2a viruses defined by the amino acid sequon: N158-Y159-T160 [5,6]; – CHO: loss of this potential glycosylation motif; Polymorphic: partial loss of the glycosylation motif.

Mutations at residues 158–160 compared with the consensus sequence for clade 3C.2a viruses are shaded in blue: dark blue shading indicates amino acid mutation relative to the consensus sequence; light blue shading indicates polymorphism relative to the consensus sequence. Two viruses were N158-Y159-K160 (– CHO) in the original patient specimen, including one that could and one that could not be HI-characterised.

[a] Final available sequence of virus isolates prior to HI characterisation is shown. Of the 234 viruses sent to the NML, 220 were re-passaged in MDCK-SIAT1 cells to attempt to improve virus titre, including 17 of 31 that could be HI-characterised and all 203 that could not be HI-characterised. Sequences for cell culture isolates as submitted from provincial reference laboratories are shown for the 13 of 14 viruses with available sequence information that could be HI-characterised without further passage in MDCK-SIAT1 cells at the NML.

[b] In the presence of 20 nM oseltamivir.

[c] One of these four viruses was T160 (i.e. + CHO) in the cell culture isolate before further MDCK-SIAT1 passage at the NML but became polymorphic after MDCK-SIAT1-passage with partial reversion to T/K160 (i.e. N158-Y159-T/K160).

[d] This virus was polymorphic for the glycosylation motif with N/D158 in the cell culture isolate before MDCK-SIAT1 passage at the NML but lost the potential glycosylation motif (i.e. became – CHO) after MDCK-SIAT1 passage with D158 (i.e. D158-Y159-T160).

Table 4

Reference haemagglutinin sequences from the GISAID EpiFlu database used to assess the 158–160 sequon in the southern hemisphere 2016 influenza A(H3N2) cell- and egg-passaged vaccine reference strain A/Hong Kong/4801/2014 (clade 3C.2a)

Segment ID	Collection date	Isolate name[a]	Originating laboratory	Submitting laboratory	Authors	Passage history	158–159–160 sequon[b]
EPI539576	26 Feb 2014	A/Hong Kong/4801/2014	Government Virus Unit	National Institute for Medical Research		MDCK-SIAT1	N-Y-T
EPI578430	1 Jan 2014	A/Hong Kong/4801/2014	Crick Worldwide Influenza Centre	Centers for Disease Control and Prevention		E5/E1	N-Y-K
EPI643118	26 Feb 2014	A/Hong Kong/4801/2014	Crick Worldwide Influenza Centre	National Institute of Infectious Diseases (NIID)	Takashita, Emi; Fujisaki, Seiichiro; Shirakura, Masayuki; Watanabe, Shinji; Odagiri, Takato	E6(Am1AI)/E1+1	N-Y-K
EPI614406	1 Jan 2014	A/Hong Kong/4801/2014 X-263	New York Medical College	Centers for Disease Control and Prevention		EX	N-Y-K
EPI614421	1 Jan 2014	A/Hong Kong/4801/2014 X-263A	New York Medical College	Centers for Disease Control and Prevention		EX	N-Y-K
EPI614414	1 Jan 2014	A/Hong Kong/4801/2014 X-263B	New York Medical College	Centers for Disease Control and Prevention		EX	N-Y-K

GISAID: Global Initiative on Sharing All Influenza Data.

[a] Originating country for all isolates displayed is Hong Kong Special Administrative Region (SAR).

[b] The consensus sequence for clade 3C.2a viruses is N158-Y159-T160, shown as N-Y-T for the cell (MDCK-SIAT1)-passaged A/Hong Kong/4801/2014 (clade 3C.2a) reference virus (first row in Table) and conferring a potential gain of glycosylation. In the egg-passaged reference A/Hong Kong/4801/2014 (clade 3C.2a) reference viruses (rows 2–6 in Table), the potential glycosylation motif is lost due to reversion to clade 3C.2 K160.

Of the 203 clade 3C.2a viruses that could not be HI-characterised, all had undergone a single further passage in MDCK-SIAT1 cells at the NML to improve viral titre. Before MDCK-SIAT1 passage, 165 of the 199 (83%) virus isolates with available sequencing information pre-MDCK-SIAT1 passage had the potential glycosylation motif associated with the clade 3C.2a consensus sequence N158-Y159-T160. After passage in MDCK-SIAT1 cells, 156 (78%) of the 199 viruses that had sequence information available post-MDCK-SIAT1 passage had the potential glycosylation motif (**Table 2**).

Among viruses with available sequencing information (n=227), the absence of the potential glycosylation motif at positions 158–160 in the final virus isolate was significantly associated with the ability to HI-characterise viruses (0 with the glycosylation motif among 28 that could be HI-characterised vs. 156 (78%) with the glycosylation motif among 199 that could not be HI-characterised; chi-square = 70.2, degrees of freedom = 1, p-value < 0.001).

Specific mutations at positions 158 and 160 of final virus isolates influencing the potential glycosylation motif prior to HI characterisation are shown in **Table 3**; the F159Y clade marker for 3C.2a viruses was conserved in all isolates.

Discussion

Similar to reports elsewhere, only a small proportion (<15%) of clade 3C.2a viruses collected through the Canadian SPSN during the 2014/15 season were able to agglutinate guinea pig erythrocytes for antigenic characterisation by HI assay [1,12,13]. All clade 3C.2a virus isolates that could be characterised were considered antigenically related to the 2015/16 vaccine strain, although more variability in HI results, particularly in relation to the egg-passaged reference virus, has been reported by other surveillance systems [1]. Our findings, however, suggest that the small proportion of clade 3C.2a viruses that could be characterised by HI assay were not representative of circulating viruses with respect to the clade-defining potential glycosylation motif at positions 158–160.

We show that cell culture passage, whether in MDCK, MDCK-SIAT1 or RMK cells, that is required for HI characterisation, can fully or partially alter the N158-Y159-T160 sequon. This sequon in circulating clade 3C.2a viruses is associated with a predicted gain of glycosylation that may be relevant for antibody binding [6]. While these findings corroborate an earlier report of this effect by the ECDC [14], here we provide direct quantification and comparison of viral genomic sequences in original patient specimens compared with culture isolates, highlighting loss of the potential glycosylation

motif as an artefact of in vitro cell culture isolation. This type of assessment has not been widely reported elsewhere, in part because most viral sequences, including those posted to public databases, are based on culture isolates and are not directly compared with primary specimens. However, understanding how viral culture impacts genetic identity before antigenic characterisation is critical to interpreting and extrapolating relatedness among vaccine and circulating strains, for the purpose of anticipating vaccine performance and for vaccine strain selection.

Limitations of our analysis include the well-recognised variability in the HI assay [17], and the small number of viruses that could be antigenically characterised for sequence comparison. Antigenic characterisation of a greater number of A(H3N2) viruses, particularly those belonging to clade 3C.2a, may be possible through use of assays that do not rely on agglutination of erythrocytes, such as neutralisation assays [14,18]. The ECDC and others have considered the N158-Y159-T160 sequon to be a potential gain of glycosylation in the majority of clade 3C.2a viruses [1,5,6]; the glycosylation potential of this motif based on the clade 3C.2a consensus sequence is 0.65 using NetNGlyc 1.0, where the threshold for glycosylation potential is 0.5 [19]. To further delineate the N-glycosylation effect of the K160T mutation, in vitro studies should be done to specifically assess the interplay between this mutation, its resulting glycosylation potential and antibody binding at antigenic sites. We show statistically significant effects of the N158-Y159-T160 sequon on the ability to characterise viruses by HI assay, but it is unclear how much this potential glycosylation motif contributes to challenges in antigenic characterisation using antibody titration assays. A proportion of clade 3C.2a viruses (43/199; 22% in this analysis, **Table 2**), as well as other A(H3N2) genetic subgroups, that lack or are polymorphic for this glycosylation motif have also been difficult to antigenically characterise by HI assay, suggesting that other factors, such as viral load and avidity to sialic acid receptors, are also likely to contribute [20]. Our goal, however, was not to investigate those factors but to assess the representativeness of clade 3C.2a viruses that could be characterised by HI assay in relation to the majority that could not be characterised, with respect to the potential glycosylation motif at pivotal antibody binding positions 158–160 of antigenic site B.

Our findings suggest that caution is warranted in extrapolating antigenic relatedness based on limited HI results for A(H3N2) clade 3C.2a viruses. Clade 3C.2a viruses have continued to predominate among A(H3N2) detections throughout the 2015 southern hemisphere influenza season and early into the 2015/16 northern hemisphere season [1,18]. Despite vaccine reformulation, clade 3C.2a viruses still differ from the northern hemisphere 2015/16 clade 3C.3a vaccine strain by 10–12 amino acids at antigenic sites, including the same N158-Y159-T160 glycosylation motif that distinguished them from the 2014/15 vaccine [2]. For the southern hemisphere's 2016 influenza season, the WHO has recommended change to an A/Hong Kong/4801/2014(H3N2)-like (clade 3C.2a) representative vaccine virus [18], for which the egg-passaged reference strain bears K160 rather than T160 and thus also seems to have lost the potential glycosylation motif (**Table 4**). Clarifying the significance of the N158-Y159-T160 potential glycosylation motif in circulating clade 3C.2a strains thus remains critical to the interpretation of antigenic relatedness and to expectations of vaccine-induced antibody protection, for which ongoing epidemiological monitoring of VE will be important.

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Conflict of interest

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Authors' contributions

Principal investigator (epidemiology): GDS (Québec); JAD (Alberta); DMS (National and British Columbia); ALW (Ontario). Principal investigator (laboratory): SD (Alberta); JBG (Ontario); MK (British Columbia); CM (Québec); NB and YL (national). Data analysis: CC, AE, SS, DMS. Preparation of first draft: DMS. Draft revision and approval: all.

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