Appendix B. Laboratory methods: additional details

Viruses

Age-based study of antibody in banked/residual sera

For the age-based sero-survey, a BC isolate of the pH1N1 virus A/California/7/2009 was used after passage 3 (P3) in MDCK cells for the hemagglutination inhibition (HI) assay and P2 for the microneutralization assay. Whole genome sequencing and translation of the P3 virus indicated that it was identical to the World Health Organization (WHO) reference strain at receptor binding sites of hemagglutinin (HA). The BC isolate differed from the WHO reference strain at two non-antigenic regions (defined according to Ndifon et al for HA [1] and Colman et al for NA [2]) of HA (Ser220Thr and Ile338Val) and at eight other sites: PB2 (Val212Ile), PA (Pro224Ser), NP (Val101Ile, Leu122Gln), NA (Val106Ile, Asn248Asp), M1 (Ala227Thr), NS1 (Ile123Val). Note that the residue numbering scheme used herein begins with the N-terminal methionine residues and refers to unprocessed protein precursors that include signal peptides. To translate HA residue numbers to the H1 scheme, subtract 17 from the residue numbering used here; to translate to the H3 scheme, subtract 14.

Seasonal H1N1 (sH1N1) and H3N2 reference viruses were obtained from the National Microbiology Laboratory (NML) in Winnipeg, Canada including A/Brisbane/59/2007 (sH1N1) (used after P2 in MDCK cells) and A/Brisbane/10/2007 (H3N2) (used after P3 in MDCK cells) and were partially sequenced. Residues 11-534 of the NML sH1N1 hemagglutinin were sequenced after P2 and differed from the WHO reference strain A/Brisbane/59/2007 by one amino acid in antigenic region B (Asp/Ile203Asn). The NML sH1N1 (P2) neuraminidase protein sequence, covering residues 1-463 of the reference strain, did not contain any mutations. The NML sH1N1 (P2) nucleoprotein was sequenced at residues 1-478 and was identical to the reference strain. The NML sH1N1 (P2) matrix proteins were sequenced entirely (M1) and near-completely (M2 residues 1-95). M1 contained a fixation of a mutation observed in the reference (Val/Ile15Ile) and M2 contained no mutations relative to the reference.

The hemagglutinin of the NML H3N2 virus was sequenced after P3 over residues 37-549 and differed from the WHO reference strain A/Brisbane/10/2007 by two amino acids in antigenic regions B (Leu210Pro) and D (Asn112Ser). The NML H3N2 (P3) neuraminidase protein sequence, spanning all 469 residues of the reference sequence, did not contain any mutations.

Birth cohort study of antibody and cytokine responses in volunteers

For the birth cohort study an A/California/7/2009 (pH1N1) reference virus was obtained from the NML and passaged three times in MDCK cells. Whole genome sequencing and translation of this P3 virus indicated that its hemagglutinin differed from the A/California/7/2009 WHO reference strain by one amino acid at antigenic region B (Leu208Ile) and a further difference at a non-antigen binding site (Thr451Asn). Two other mutations were observed: NP (Leu122Gln), M1 (Ala22Thr).

BC isolates of A/Brisbane/59/2007-like sH1N1 (P3) and A/Brisbane/10/2007-like H3N2 (P2 for HI and P3 for MN) were used in the birth cohort study and were partially sequenced. Residues 11-534 of the BC sH1N1 hemagglutinin were sequenced after P3 and differed from the WHO reference strain A/Brisbane/59/2007 at six positions: four in antigenic region B (Asn200Ser, Gly202Asn, Asp/Ile203Asn, Ala206Thr) and two in non-antigenic regions (Glu137Asp, Ala531Thr). The BC sH1N1 (P3) neuraminidase was sequenced at residues 45-401 and differed from the reference strain at four sites: Val83Met, His275Tyr, Asp354Gly, and Ans355Asp. The BC sH1N1 (P3) nucleoprotein was sequenced at residues 1-478 and was identical to the reference strain. The BC sH1N1 (P3) matrix proteins were sequenced entirely (M1) and near-completely (M2 residues 1-95). M1 contained a fixation of a mutation observed in the reference (Val/Ile15Ile) and M2 contained no mutations relative to the reference.

The hemagglutinin protein of the BC H3N2 virus spanning residues 14-539 of the WHO reference strain A/Brisbane/10/2007 was sequenced after P2 and differed by one amino acid in antigenic region E (Lys189Gln; an *A/Perth/16/2009-like* mutation) and by two amino acids at non-antigenic regions (Thr16Ala, Ile377Arg). The BC H3N2 (P2) neuraminidase was sequenced completely and differed from the reference by seven amino acids, three of which were A/Perth/16/2009-like (Asp147Asn, Ile215Val, Thr312Ile). The other mutations comprised Ile30Val, Thr148Ile, Glu221Asp, and Asp463Asn.

Deposition of viral nucleotide sequences in GenBank

Nucleotide sequences of all passaged study virus segments were deposited into GenBank under the following accession numbers: NML sH1N1 (P2): CY065747 (HA), CY065748 (NP), CY065749 (NA), CY065750 (M); NML H3N2 (P3): CY065751 (HA), CY065752 (NA); BC H3N2 (P2): CY065753 (HA), CY065754 (NA); BC sH1N1 (P3): CY065755 (HA), CY065756

(NP) CY065757 (NA), CY065758 (M); BC pH1N1 (P3): CY065759 (PB2), CY065760 (PB1), CY065761 (PA), CY065762 (HA), CY065763 (NP), CY065764 (NA), CY065765 (M), CY065766 (NS); NML pH1N1 (P3): CY065767 (PB2), CY065768 (PB1), CY065769 (PA), CY065770 (HA), CY065771 (NP), CY065772 (NA), CY065773 (M), CY065774 (NS).

Percent homology between viruses used and WHO reference strains

For each pairwise combination of study viruses and reference strains, the pairwise identity and number of mutations between protein sequences was calculated (Table B1) for full-length proteins and for extractions of the HA and NA antigenic regions. H3 HA antigenic regions were extracted according to Ndifon et al [1] and N2 NA antigenic regions according to Colman et al [2]; these regions were then mapped to homologous sites in H1 and N1 proteins using the MUSCLE multiple alignment algorithm [3] as implemented in Geneious [4]. MUSCLE in Geneious was also used to calculate pairwise identity between viral proteins. The HA and NA sequences from the A/Brevig Mission/1/1918 viruses were included in pairwise comparisons for reference.

Hemaglutination inhibition (HI) assay

All sera were frozen at ≤-20˚C until prepared for testing. For the HI assay, sera were treated with receptor destroying enzyme to remove non-specific agglutinins. Sera from which non-specific agglutinins were not removed were further hemadsorbed. Sera were serially diluted beginning at 1:10 with PBS and reacted with 4 HI units of antigen (infected MDCK cell lysate) for 45 minutes. 25 µL of antigen were incubated with 25 µL of each serum dilution for 30 minutes. To each mixture 50 µL of 0.5% turkey erythrocytes were added, and after mixing, the preparations were incubated for 30 minutes. Results were recorded by photography. The HI titre was designated as the inverse of the highest dilution to still show some hemagglutination inhibition. All sera were assayed in duplicate.

Microneutralization (MN) assay

All sera were frozen at ≤-20˚C until prepared for testing. For the MN assay, the viral titres were determined by tissue culture infective dose-50 based on the Karber method[5]. The sera were serially diluted beginning at 1:10 and to each dilution, 100 infectious units of virus

were added. The plates were incubated for 2 hours at 37°C to allow for virus-antibody interaction. The contents of each well were then transferred onto microtitre plates with confluent monolayers of MDCK cells. After 3 hours of further incubation at 37°C, the medium in each well was removed and replaced with fresh medium (MegaVir, HyClone, Utah) containing TPCK-treated trypsin. The plate was then again incubated at 37°C and monitored for the appearance of cytopathic effects (CPE) on days 3 and 5. The MN titre was defined as the serum dilution in the well immediately preceding the wells with CPE. MN was conducted in duplicate up to two times until intra-assay concordance was achieved (≤2-fold) and GMTs reported.

Preparation of virus for CMI assays

Viruses were grown on MDCK cell monolayers in the presence of trypsin as outlined above. Virus was purified from infected cell lysates by differential ultracentrifugation (3,000 x g for 20 minutes twice followed by 100,000 x g for 90 minutes). The pellet was resuspended in Megavir medium and virus titrated by HA using turkey erythrocytes as outlined above.

References

1. Ndifon W, Wingreen NS, Levin SA. Differential neutralization efficiency of hemagglutinin epitopes, antibody interference, and the design of influenza vaccines. Proc Natl Acad Sci U S A. 2009;106:8701-6.
2. Colman PM, Varghese JN, Laver WG. Structure of the catalytic and antigenic sites in influenza virus neuraminidase. Nature 1983;303:41-4.
3. Drummond AJ, Ashton B, Cheung M, et al. Geneious v4.8. 2009; http://www.geneious.com/
4. Edgar RC. MUSCLE: a multiple sequence alignment method with reduced time and space complexity. BMC Bioinformatics. 2004;5:113.
5. Karber G. Beitrag zur Kollektiven Behandling Pharmakologischer Reihenversuche, Arch Exp. Path, Pharmacol.1931;162:480-3.

Table B1. Pairwise percent identity and (number of amino acid mutations) in surface and internal proteins across viruses used in laboratory assays against each other, WHO reference strains, and the 1918 H1N1 virus

Comparison of pH1N1 viruses: full-length proteins

	percent identity (number of mutations)					
Protein	BC pH1N1 vs WHO pH1N1	NML pH1N1 vs WHO pH1N1	BC pH1N1 vs NML pH1N1	BC pH1N1 vs BC sH1N1	BC pH1N1 vs 1918 H1N1	NML pH1N1 vs 1918 H1N1
PB2	99.9 (1)	100 (0)	99.9 (1)	-	96.6 (26)	96.7 (25)
PB1	100 (0)	100 (0)	100 (0)	-	96.3 (28)	96.3 (28)
PA	99.9 (1)	100 (0)	99.9 (1)	-	96.6 (24)	96.5 (25)
HA	99.6 (2)	99.6 (2)	99.3 (4)	79.0 (118)*	83.5 (67)*	83.7 (66)*
NP	99.6 (2)	99.8 (1)	99.8 (1)	89.7 (49)*	94.6 (27)	94.4 (28)
NA	99.6 (2)	100 (0)	99.6 (2)	80.7 (90)*	87.4 (59)	87.4 (59)
M1	99.6 (1)	99.6 (1)	99.2 (2)	-	95.2 (12)	95.2 (12)
M2	100 (0)	100 (0)	100 (0)	-	91.8 (8)	91.8 (8)
NS1	99.5 (1)	100 (0)	99.5 (1)	-	84.9 (33)*	85.4 (32)*
NS2	100 (0)	100 (0)	100 (0)	-	90.1 (12)	90.1 (12)

Comparison of pH1N1 viruses: antigenic regions only

Protein	BC pH1N1 vs WHO pH1N1	NML pH1N1 vs WHO pH1N1	BC pH1N1 vs NML pH1N1	BC pH1N1 vs BC sH1N1	BC pH1N1 vs 1918 H1N1	NML pH1N1 vs 1918 H1N1
HA	100 (0)	99.3 (1)	99.3 (1)	53.0 (62)	73.9 (35)*	73.1 (36)*
NA	100 (0)	100 (0)	100 (0)	59.4 (13)	87.5 (4)	87.5 (4)

Comparison of sH1N1 viruses: full-length proteins

Protein	BC sH1N1 vs WHO sH1N1	NML sH1N1 vs WHO sH1N1	BC sH1N1 vs NML sH1N1	BC sH1N1 vs 1918 H1N1	NML sH1N1 vs 1918 H1N1
HA	98.9 (6)*	99.8 (1)*	99.0 (5)*	82.1 (71)*	82.1 (71)*
NA	98.9 (4)*	100 (0)*	98.9 (4)*	84.8 (55)*	86.8 (61)*
NP	100 (0)*	100 (0)*	100 (0)*	93.3 (32)*	93.3 (32)*
M1	100 (0)	100 (0)	100 (0)	96.0 (10)	96.0 (10)
M2	100 (0)*	100 (0)*	100 (0)*	85.3 (14)*	85.3 (14)*

Comparison of sH1N1 viruses: antigenic regions only

Protein	BC sH1N1 vs WHO sH1N1	NML sH1N1 vs WHO sH1N1	BC sH1N1 vs NML sH1N1	BC sH1N1 vs 1918 H1N1	NML sH1N1 vs 1918 H1N1
HA	97.1 (4)	99.3 (1)	97.0 (4)	66.4 (45)*	66.4 (45)*
NA	100 (0)	100 (0)	100 (0)	67.9 (10)	68.8 (10)

Comparison of H3N2 viruses: full-length proteins

Protein	BC H3N2 vs WHO '07 H3N2	NML H3N2 vs WHO '07 H3N2	BC H3N2 vs NML H3N2	BC H3N2 vs WHO '09 H3N2	NML H3N2 vs WHO '09 H3N2
HA	99.4 (3)*	99.6 (2)*	99.2 (4)*	99.0 (5)*	98.2 (9)*
NA	98.5 (7)	100 (0)	98.5 (7)	99.1 (4)	99.4 (3)

Comparison of H3N2 viruses: antigenic regions only

Protein	BC H3N2 vs WHO '07 H3N2	NML H3N2 vs WHO '07 H3N2	BC H3N2 vs NML H3N2	BC H3N2 vs WHO '09 H3N2	NML H3N2 vs WHO '09 H3N2
HA	99.2 (1)	98.5 (2)	97.7 (3)	96.2 (5)	93.9 (8)
NA	100 (0)	100 (0)	100 (0)	100 (0)	100 (0)

Note. pH1N1 = pandemic H1N1 virus. sH1N1 = seasonal H1N1 virus. BC refers to BC isolates used in present study (see Appendix B). NML refers to NML isolates used in present study (see Appendix B). WHO refers to posted vaccine reference strains; WHO pH1N1=A/California/7/2009; WHO sH1N1=A/Brisbane/59/2007; WHO '07 H3N2=A/Brisbane/10/2007; WHO '09 H3N2= A/Perth/16/2009; 1918 H1N1=A/Brevig Mission/1918. Asterisks indicate pairwise comparison in which one or both segments were not fully sequenced.