

International laboratory comparison of influenza microneutralization assays for A(H1N1)pdm09, A(H3N2), and A(H5N1) influenza viruses by CONSISE

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1 **Submitted as a full-length paper to *Clinical and Vaccine Immunology***
2 **An international laboratory comparison of influenza microneutralisation assay**
3 **protocols for A(H1N1)pdm09, A(H3N2) and A(H5N1) influenza A viruses by CONSISE**
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24 **Running Title:** Comparing MN assay methods
25

26 **Abstract – 205 words (250 allowed)**

27 The Microneutralisation assay is commonly used to detect antibodies to influenza virus and
28 multiple protocols are used worldwide. These protocols vary in the incubation time of the
29 assay as well as in the order of specific steps and even within protocols there are often further
30 adjustments in individual laboratories. The impact these protocol variations have on influenza
31 serology data is unclear. Thus a laboratory comparison of the 2-day ELISA and 3-day
32 hemagglutination (HA) microneutralisation (MN) protocols, using A(H1N1)pdm09,
33 A(H3N2) and A(H5N1) viruses, was performed by the CONSIZE Laboratory Working
34 Group. Individual laboratories performed both assay protocols, on multiple occasions, using
35 different serum panels. Thirteen laboratories from around the world participated. Within each
36 laboratory, serum sample titres for each assay protocol were compared to determine the
37 sensitivity of each assay and between replicates to assess the reproducibility of each protocol,
38 for each laboratory. There was good correlation between the results obtained using the two
39 assay protocols in most laboratories, indicating these assays may be interchangeable for
40 detecting antibodies to the influenza A viruses included in this study. Importantly,
41 participating laboratories have aligned their methodology to the CONSIZE Consensus 2-day
42 ELISA and 3-day HA MN assay protocols to enable better correlation of these assays in the
43 future.

44

45 **Keywords:** influenza; serology; CONSIZE; microneutralisation assay; pandemic; A(H5N1);
46 A(H1N1)pdm09; A(H3N2)

47

48 Introduction

49 Following infection with influenza viruses, most people develop antibodies specific to
50 the infecting virus that can be measured by serological assays. These antibodies can be
51 detected in the majority of people 2-3 weeks after symptom onset and persist for months (1,
52 3, 7, 13). Thus serology can confirm past infection in the absence of clinical symptoms or
53 virological data, detecting most symptomatic and asymptomatic infections(6).

54 In 2011, an international partnership termed 'CONSIDE', the Consortium for the
55 Standardization of Influenza Seroepidemiology, was created out of a need identified during
56 the 2009 pandemic, for timely seroepidemiological data to better estimate pandemic virus
57 infection severity and attack rates and to inform policy decisions. CONSIDE is comprised of
58 individuals from various organisations, with free membership. The activities of CONSIDE are
59 performed by two inter-linked Working Groups, Laboratory and Epidemiology, and a
60 Steering Committee. The focus of the Laboratory Working Group is to improve serological
61 assay comparability and standardisation through consensus assay development, comparative
62 laboratory testing and quality assurance (12)([www. https://conside.tghn.org](http://www.conside.tghn.org)).

63 The main serological assays to detect antibodies to influenza virus are the
64 Hemagglutination Inhibition (HI) assay and the Microneutralisation (MN) assay. The HI
65 assay detects antibodies that block the influenza virus hemagglutinin binding to sialic acid-
66 linked residues on red blood cells (RBC), whilst the MN assay detects functional antibodies
67 primarily directed towards the hemagglutinin that prevent infection of cells in tissue culture
68 (reviewed in (5, 17)). There are various forms of the MN assay used in laboratories around
69 the world, such as the 2-day ELISA protocol (8, 17), 3-day hemagglutination (HA) protocol
70 (16) and 7-day HA protocol(4, 9). For the purposes of seroepidemiology, the shorter
71 protocols of 2 and 3 days are preferred. The 2- and 3-day MN assays measure antibodies to
72 hemagglutinin, yet differ in preparation of cell monolayers for infection as well as detection

73 of virus infection. Cells are plated with the virus:serum mixture for the 2 day MN assay,
74 whilst a pre-formed cell monolayer is used for the 3-day MN assay. The 2-day MN assay
75 detects nucleoprotein in infected cells (8), whilst the 3-day assay measures hemagglutinating
76 virus in the culture medium or CPE in the cell monolayer. Although there have been some
77 direct comparisons between serological assays performed by multiple laboratories (9, 10, 14,
78 15), the impact of various MN assay protocols on the determination of serological titres is
79 unknown. Therefore, the aim of this study was to assess the intra-laboratory variability and
80 sensitivity of the 2-day ELISA MN assay and the 3-day HA MN assay for detecting
81 antibodies to A(H1N1)pdm09 virus and, as an extension, A(H3N2) and A(H5N1) influenza
82 viruses. The study was performed by the CONSISE Laboratory Working Group members
83 (see Appendix).

84

85 **Methods**

86

87 **Reagents used in study**

88 Laboratories were required to supply their own reagents, virus stocks, MDCK cell lines and
89 appropriate cell culture media for the study. Wildtype or reassortant viruses were used: the
90 A(H1N1)pdm09 strains were antigenically similar to the A/California/7/2009 vaccine strain;
91 the A(H3N2) strains were antigenicity similar to the A/Perth/16/2009 or A/Victoria/361/2011
92 vaccine strains. A representative A(H5N1) virus from a clade that was recognised by the
93 laboratory's serum panel was used. Serum panels contained approximately 10 test samples
94 (sera or plasma), comprising low, medium and high titre antibody levels. Sera were from
95 seroepidemiology studies, vaccine studies and ferret sera (to obtain high titre serum in some
96 laboratories), and were supplied by each participating laboratory.

97

98

99 **Development of consensus 2-day ELISA and 3-day MN protocols**

100 Parameters and variables for the 2-day ELISA (17) and the 3-day HA (11, 16) MN assays
101 were listed. Laboratories within CONSISE shared their protocols for either, or both, MN
102 assays and listed their preferred variables for each parameter identified. Data were collected
103 anonymously, collated and used to develop the consensus protocols.

104

105 **Consensus 2-day ELISA and 3-day HA MN assays**

106 The 2-day ELISA MN assay was to be performed as described by (8, 17) whilst the 3-day
107 HA MN assay was to be performed as described by (11, 16). Laboratories were required to
108 use the specified parameters listed in the CONSISE consensus protocols (**Tables 1 and 2**).
109 Cell culture conditions, virus and serum panels varied between laboratories. The reciprocal of
110 the highest dilution whereby 50% infection was prevented was recorded as the titre for each
111 serum sample.

112

113 **Design of study – laboratory assay comparison**

114 Individuals who are members of CONSISE were invited to participate in the experimental
115 laboratory comparative study. Thirteen laboratories agreed and were assigned a code letter
116 from A-M, not representing the order of listing of participants in the Appendix. Eleven
117 laboratories took part in the initial A(H1N1)pdm09 study (Labs A-K), seven in the A(H3N2)
118 study (Labs A, C, D, F, I, K, L) and three in the A(H5N1) study (Labs H, L, M). Overall
119 twelve laboratories provided data that could be included in the analyses (Labs A-L). Each
120 laboratory was required to assay A(H1N1)pdm09, A(H3N2) or A(H5N1) antibody levels in
121 their panel of sera on at least three separate occasions using both the CONSISE consensus
122 MN assay protocols: 2-day ELISA and 3-day HA.

123

124 **Statistical analysis**

125 All analyses were based on the titres reported by the participants. To enable comparison of
126 assays for each laboratory, the geometric mean titre (GMT) was calculated across runs and
127 replicates to give a single value for each sample for each MN assay method. To calculate the
128 overall ratios between the assays for each laboratory, the ratio of the 3-day titre (detected by
129 HA or cytopathic effect (CPE)) to the 2-day ELISA MN titre was calculated for each sample.
130 The GMT was then calculated for all samples in the serum panel for each laboratory. For the
131 purpose of calculations, negative titres reported as <10 were substituted by '5', while high
132 titres reported as \geq were assigned the next 2-fold titre, e.g.. ' ≥ 1280 ' was assigned '2560'.
133 Correlations in results between assay methods for the panels of serum samples were
134 calculated using Spearman rank correlations.

135

136 **Results**

137

138 **Development of Consensus protocols for the MN assays**

139 We assessed the similarities between methodology used in ten laboratories for the 2-day
140 ELISA and the 3-day HA MN assays. Parameters were highly consistent between
141 laboratories for the 2-day ELISA MN assay method, and closely followed published methods
142 (8, 17). There was less consistency between the 3-day HA MN assay methods, particularly in
143 number of sample replicates performed and determination of the endpoint titre (50 % or
144 100% neutralisation). There was variability in both assays for cell culture conditions (data not
145 shown). To facilitate greater comparability between laboratories, we developed consensus
146 protocols for the 2-day ELISA and 3-day HA MN assays by discussion and agreement at
147 CONISE meetings ((2) (**Tables 1** and **2**)). Parameters were classified as either required or

recommended, based on their importance in the interpretation of the assay titres. Required parameters included serum dilutions and reporting of sample titres, assay incubation times and endpoint calculation methods. Recommended parameters listed appropriate variables for use.

Data received

A(H1N1)pdm09 MN assays: Ten laboratories returned data for both assays. ELISA was used for detection of all 2-day MN assays. For the 3-day MN assay, 7 laboratories used HA only as the detection method (turkey or guinea pig RBC were used), 2 laboratories used CPE only and 2 laboratories used both HA and CPE detection methods. Both HA and CPE detection methods were assessed. Three laboratories performed multiple additional detection methods for one or both of the MN assays, but these data have not been included in the analysis. An eleventh laboratory did not perform the 2-day ELISA MN assay and returned data for only the 3-day HA MN assay (laboratory K). This laboratory shared a serum panel with laboratory F which performed both assays. The 3-day HA MN assay titres from laboratory K were compared with the 2-day ELISA MN assay titres from laboratory F. Laboratories G and J performed each assay twice, rather than three times; laboratory I performed the 3-day MN assays twice.

A(H3N2) MN assays: Data were received from seven laboratories. All laboratories used ELISA for detection of the 2-day MN assay and HA (turkey or guinea pig RBC) for detection of the 3-day HA MN assay and two laboratories also sent corresponding titres detected by CPE. Laboratory K only performed the 3-day HA MN assay and shared a serum panel with laboratory F.

173 **A(H5N1) MN assays:** Data were received from three laboratories. The results from
174 laboratory M were negative for all of the serum samples for all tests. No further analysis was
175 possible for this laboratory. Laboratories H and L used horse or goose RBC to read out the 3-
176 day HA MN assay.

177

178 **Reproducibility within laboratories: comparison of replicate tests**

179 Laboratories performed an internal comparison of assay protocols using their own serum
180 panels. The titres within each laboratory for each sample across replicate tests were
181 compared. Detecting antibodies to A(H1N1)pdm09 virus, there was good reproducibility of
182 the 2-day ELISA MN assays for the majority of the laboratories, with over 80% of the
183 laboratories with replicate tests differing by ≤ 2 -fold, whilst laboratories B and D had 10-30%
184 of replicate titres differing by >2 -fold (**Figure 1A**). No laboratories had replicate tests
185 differing by >4 -fold (**Figure 1B**). For the 3 day MN assays, the variability differed depending
186 on the assay detection method. When detecting by HA, 4 laboratories had replicates differing
187 by >4 -fold, yet this was only for very few samples (one sample out of 10-12 samples, 8.3-10
188 %), whilst when detecting by CPE, two laboratories had replicate tests differing by >4 -fold
189 (two samples of 10 (20 %) and 7/12 samples (58 %)) (**Figure 1B**). Six laboratories had
190 replicates differing by >2 -fold by HA detection (7.5-40 %), whilst three laboratories had tests
191 differing by >2 -fold when detected by CPE (10-91.7 %) (**Figure 1A**). Laboratory A showed
192 high variability between replicates when detecting the 3-day MN titres by CPE (91.7 %) as
193 two replicate assays were comparable, whilst the third assay was inconsistent (data not
194 shown). In seven laboratories (C, D, E, F, G, J, K), there were no replicates that differed by
195 more than 2-fold.

196 For studies detecting antibodies to A(H3N2) and A(H5N1) viruses, reproducibility was
197 also good, with three instances of replicates that differed by greater than 4-fold, though this

198 was for a small number of samples for each laboratory (10-20%). All other laboratories had
199 replicates that differed by ≤ 4 -fold (**Figure 1C and D**).

200

201 **Relationship between 2-day ELISA MN assay and 3-day HA and CPE MN assays for**
202 **test serum panels**

203 Titres for individual test serum panels were compared for each laboratory between the two
204 assays. Comparing the titres obtained using the 2-day ELISA and the 3-day HA MN assays
205 for studies detecting antibodies to A(H1N1)pdm09 virus (**Figure 2A**), seven of the nine
206 laboratories had individual correlation co-efficients that were above 0.9 (good) and two of the
207 nine laboratories did not (**Table 3**). Two laboratories (G and H) had low correlation between
208 the assays (0.580 and 0.638, respectively), as the 3-day MN HA assay gave narrow response
209 ranges compared to the 2-day ELISA MN assay. Comparison of titres between the 2-day
210 ELISA MN assay and the 3-day MN assay detected by CPE showed higher overall
211 consistency between the assays (**Figure 2B, Table 3**).

212 The equivalent correlations were determined for comparison of assays detecting
213 antibodies to A(H3N2) (**Figures 2C, D**) and A(H5N1) (**Figures 2E, F**). For assays detecting
214 antibodies to A(H3N2) virus, the majority of laboratories obtained good correlation between
215 the 3-day HA and the 2-day ELISA MN assays (range 0.865-0.966 (**Table 3**)). Laboratory L
216 had a poor correlation with co-efficient of 0.439 as both of the assays gave a narrow titre
217 range across the serum panel (<10-160). There were only two laboratories that had data
218 detecting antibodies to A(H5N1) virus and they both showed reasonable correlation between
219 the assays (**Table 3**). Laboratory L had much better correlation with the assays detecting
220 antibodies to A(H5N1) virus than A(H3N2) virus.

221

222

223 **Overall relationship between assays for each laboratory**

224 The ratio between titres for the 2-day ELISA and 3-day MN assays for the serum panels was
225 calculated to assess whether a consistent relationship between the performances of assays
226 could be observed for each laboratory (**Table 4**). Many of the ratios indicated average titres
227 within a 2-fold range between methods (i.e. ratios between 0.5 and 2.0), representing
228 reasonable agreement in assay sensitivity. For assays detecting antibodies to A(H1N1)pdm09,
229 laboratories F and G had lower 3-day HA MN titres compared to their 2-day ELISA MN
230 titres. For laboratory G, the 3-day HA MN assay gave negative or low titres for all serum
231 panel samples. Laboratories I and K had much higher 3-day MN titres compared to the 2-day
232 ELISA MN assay. The ratios between assays were different for the laboratories that also
233 participated in the comparison studies detecting antibodies to A(H3N2) or A(H5N1). For the
234 comparison assays detecting A(H3N2) antibodies, laboratories D and K also had much higher
235 3-day MN titres compared to the 2-day ELISA MN assay. For comparison assays detecting
236 A(H5N1) antibodies, laboratory H had much higher 3-day HA MN assay titres compared to
237 the 2-day ELISA MN assay titres (**Table 4**).

238

239 **Analysis for bias within the study**

240 Potential factors for bias were assessed. Although each laboratory had a preferred MN assay
241 (2 day ELISA, 3 day HA or 3 day CPE, indicated in **Table 3**), overall this did not seem to
242 affect the correlation between assays. However, for laboratories where the titres from the two
243 assays did not correlate well (A(H1N1)pdm09 virus for laboratories G, H; A(H3N2) virus for
244 laboratory L, **Table 3**) the 3-day MN assay showed less variation in titres (i.e. had less
245 discriminating power) (Figure **2A** and **C**, respectively). Yet there was also variability in
246 correlation for different viruses within the same laboratory (**Table 3**), indicating this effect
247 may be virus specific or due to experience, as the studies were performed consecutively

(A(H1N1)pdm09, then A(H3N2)/A(H5N1)). However, for laboratories with markedly different overall titres between assays (laboratories F, H, I and K, **Table 4**) there was no relationship between the preferred assay and the titre achieved. Most laboratories used sera from adults (10 of 11) and wildtype influenza viruses (8 of 11); neither showed any effect on assay comparability. The ratio of HA/TCID₅₀ for the virus stock from each laboratory was assessed and there was no clear relationship, suggesting there was no bias due to the presence of interfering virus particles (HA titres range - 16-1280, median - 128; log₁₀ TCID₅₀/ml range - 4.5-7, median - 6). Overall, there was no clear indication of major bias in the study.

Discussion

Upon emergence of a novel influenza virus, seroepidemiological data are critical in understanding the spread and attack rate of the virus to form the base of pandemic risk and severity assessments. Serology also can identify groups susceptible to a novel influenza virus in a population. Understanding the impact of different MN assay protocols would strengthen these estimates for policy decisions. Our comparison of MN assay methodology indicates that there is good correlation between the 2-day ELISA and 3-day HA MN assays for detection of antibodies to A(H1N1)pdm09 virus, in most laboratories. These findings were confirmed in an extension to this study with A(H3N2) and A(H5N1) viruses. Overall, there is potential for either assay to be used. Importantly, through participating in this study, laboratories have aligned their methodology to the CONSIZE consensus assays described, harmonising protocols for the 2-day ELISA and 3-day HA MN assays internationally.

Our intra-laboratory assessment demonstrated the 2-day ELISA and 3-day HA MN assays were largely reproducible and comparable. The 2-day MN assay is read out by ELISA using spectrophotometry, which is objective, whilst the 3-day MN assay that is read out by HA and CPE requires more experience and training. In addition, as our study required

273 participating laboratories to perform the assays on three separate occasions, different
274 preparations of RBC for the replicate 3-day HA MN assays were likely. As a two-fold range
275 between titres for the same sample is considered acceptable for serological studies, the assays
276 were overall highly reproducible on different days, with all laboratories having >97% of
277 samples with titres within a four-fold difference for the 2-day ELISA, 92% for the 3-day HA
278 and 92% for the 3-day CPE MN assays.

279 Importantly, in over half of the laboratories, there was very good correlation between
280 the 2-day ELISA and 3-day HA MN assays when a panel of sera was tested. This suggests
281 there is no inherent difference between the different assays, despite their different readouts.
282 Thus there is no underlying scientific reason that the different MN assay formats cannot be
283 compared when detecting antibodies to A(H1N1)pdm09, A(H3N2) or A(H5N1) viruses.

284 However, three laboratories did have poor correlation between the assays which may be
285 related to experience in, and performance of, one particular method. Seven laboratories were
286 experienced in the 2-day ELISA MN assay, whilst 6 laboratories were experienced in the 3-
287 day HA MN assay before commencing this study. Poor correlation between assays was more
288 likely in those laboratories inexperienced with the 3-day HA MN assay, indicating that
289 training in HA or CPE readout might be required. From the MN assay comparison performed
290 here, we anticipate that as laboratories gain experience in both assays, the correlation
291 between the titres obtained for the 2-day ELISA and 3-day HA MN assays will improve. A
292 mentoring system will be established in subsequent international comparison studies
293 performed by CONSISE whereby laboratories who are learning an assay will be assisted by
294 local 'experienced' laboratories. We anticipate that this collaborative assay development will
295 encourage rapid and more comparable data between assays and laboratories in the future.

296

297 It is notable that although we were able to standardise many assay-specific variables in
298 this study, such as virus concentration, incubation times, serum dilution and end-point
299 determination, some factors are impossible to standardise. Cell culture conditions are
300 laboratory-specific and may differ by cell line, media supplements and incubation
301 temperatures. Cell culture conditions are often optimised for the variety of viruses and
302 experiments performed in a laboratory as well as the availability of reagents, thus cannot be
303 prescriptive.

304

305 A limitation of the present study is that test serum panels were not shared with each
306 laboratory. As our study compared assay protocols, rather than the performance of different
307 laboratories, this is acceptable. Thus the impact of using consensus assay protocols on inter-
308 laboratory variability could not be examined thoroughly. In a future study being planned by
309 the CONSIZE Laboratory Working Group, shared serum panels will be tested for antibodies
310 to A(H1N1)pdm09 virus using consensus 2 day ELISA and 3-day MN Assays and a
311 consensus HI Assay in comparison with use of local assay protocols.

312

313

314

315 **Appendix**

316 **Study Participants:** Jaccqueline Katz, Xiuhua Lu, Min Levine, Vic Veguilla, Feng Liu,
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340

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351

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356 following patents: Preparation and use of recombinant influenza A virus M2 construct
357 vaccine, Materials and methods for respiratory disease control in canines and An effective
358 vaccine against pandemic strains of influenza viruses issued.

359

360

361

362

363 **Figure Legends**

364

365 **Figure 1.** Reproducibility within laboratories of serology assay results for assays detecting
366 antibodies to A(H1N1)pdm09 (**A, B**) and A(H3N2) and A(H5N1) (**C, D**) viruses. Graphs
367 show the proportion (%) of replicate assays differing by >2-fold (**A, C**) and >4-fold (**B, D**)
368 for 2 day ELISA MN Assay (black bar), 3 day MN Assay detected by HA (white bar) and
369 CPE (striped bar) for each participating laboratory for all sera. ND indicates where the assay
370 or detection method was not performed.

371

372 **Figure 2.** Relationship between test sample titres for antibodies to A(H1N1)pdm09 (**A, B**),
373 A(H3N2) (**C, D**) or A(H5N1) (**E, F**) viruses determined by 2-day ELISA MN Assay and 3
374 day MN Assay detected by HA (**A, C, E**) or CPE (**B, D, F**). Each laboratory is represented by
375 a colour.

376

377

378

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Table 1. CONSIDE Consensus 2-day ELISA MN Assay for detecting antibodies to A(H1N1)pdm09 virus (based on [1, 2])

Parameter	Required Parameter	Recommended parameter
A. Stock Virus preparation		
Cell substrate for virus growth		Day 10 embryonated eggs
Stock virus infectivity and method of determination		At least 10^6 TCID ₅₀ /ml, read by ELISA
Stock storage		Aliquots of bulk virus preparation
B. Sera preparation		
Storage of sera following receipt		-70 °C, -20 °C, 4 °C, 1-2 freeze thaw cycles in testing laboratory
Pre-assay treatment of sera		Heat treatment 56 °C for 30 min, undiluted in media
Initial sera dilution	1:10	-
Sample type		Sera only or plasma only
C. Virus preparation		
Final virus concentration per well	100TCID ₅₀	-
Volume of virus solution added per sample	50 µl	-
Virus/serum mix incubation		1h at 37 °C
Calculated starting sera dilution	1:10 excluding cell culture	-

	volume	
D. Cell preparation		
Preparation of cells		Cell suspension
Cell type used		MDCK ('Salisbury'), MDCK-SIAT1
Assay diluent/culture media		Coon's/Dulbecco's Modified Eagles with 1% BSA/FCS, laboratory preferred media
E. Assay set-up		
Incubation time of assay to endpoint reading	18 -22h	
Incubation conditions		35-37 °C, 5% CO ₂
# of sample replicates		Replicates preferred if available
F. Endpoint estimation		
Endpoint determination		Viral antigen detection by ELISA using anti-nucleoprotein antibody (clone)
Endpoint calculation method	50% neutralisation	

Table 2. CONSIDE Consensus 3-day HA MN Assay for detecting antibodies to A(H1N1)pdm09 virus

Parameter	Required Parameter	Recommended parameter
A. Stock Virus preparation		
Cell substrate for virus growth		Day 10 embryonated eggs, MDCK cells, MDCK-SIAT1 cells
Stock virus infectivity and method of determination		At least 10^6 TCID ₅₀ /ml, read by RBC agglutination
Stock storage		Aliquots of bulk virus preparation
B. Sera preparation		
Storage of sera following receipt		-70 °C, -20 °C, 4 °C, 1-2 freeze thaw cycles in testing laboratory
Pre-assay treatment of sera		Heat treatment 56 °C for 30 min, undiluted in media
Initial sera dilution	1:10	-
Sample type		sera only or plasma only
C. Virus preparation		
Final virus concentration per well	100TCID ₅₀	
Volume of virus solution added per sample/well		50 µl, 100 µl, 200µl
Virus/serum mix incubation		1h at 37 °C
Virus/serum mix incubation on cell monolayer		1h at 37 °C
Calculated starting sera dilution	1:10 excluding virus	-

	volume	
D. Cell preparation		
Preparation of cells		preformed monolayer
Cell type used		MDCK (ATCC), MDCK ('Salisbury'), MDCK-SIAT1
Assay diluent		Coon's/Dulbecco's Modified Eagles, with trypsin (1/2 µg/ml), laboratory preferred media
Cell infection media		Coon's/Dulbecco's Modified Eagles, with trypsin (1/2 µg/ml), laboratory preferred media
E. Assay set-up		
Incubation time of assay to endpoint reading	3 days	
Incubation conditions		35-37 °C, 5% CO ₂
# of sample replicates		Replicates preferred if available
F. Endpoint estimation		
Endpoint determination		turkey/guinea pig RBC agglutination, CPE
Endpoint calculation method	50% neutralisation	

Table 3. Correlation of titres for test samples between assays by laboratory. For each laboratory, Spearman rank correlation coefficients were calculated to compare the 2-day ELISA MN against the 3-day HA or CPE MN assay.

Laboratory	Correlation of 2-day MN to 3-day MN						Preferred assay
	A(H1N1)pdm09		A(H3N2)		A(H5N1)		
	3-day	3-day	3-day	3-day	3-day	3-day	
	HA	CPE	HA	CPE	HA	CPE	
A	0.966	0.901	0.865				2 day
B	0.976						2 day
C	0.992		0.966				3 day
D		0.892	0.890	0.898			3 day
E	0.944						2 day
F	0.965		0.966				3 day
G	0.580						2 day
H	0.638	0.738			0.883	0.908	2 day
I	0.970		0.954	0.901			2 day
J		0.944					3 day
K ^a	0.931		0.942				3 day
L			0.439		0.833		both

^aTitres for 2-day ELISA MN from laboratory F, as laboratories F and K shared serum panels.

Table 4. Ratios between 2-day ELISA MN Assay and 3-day MN Assay detected by HA and CPE.

Laboratory	Average Ratio of 3-day MN titre to 2-day MN titre					
	A(H1N1)pdm09		A(H3N2)		A(H5N1)	
	3-day HA	3-day CPE	3-day HA	3-day CPE	3-day HA	3-day CPE
A	2.4	1.5	1.9			
B	0.9					
C	0.8		1.5			
D		2.0	3.7	3.8		
E	1.0					
F	0.3		1.3			
G	0.1					
H	1.5	1.6			5.8	6.3
I	5.22		0.88	0.87		
J		1.2				
K ^c	2.4 ^a		3.2			

L 1.1 0.4

^aTitres for 2-day ELISA MN from laboratory F, as laboratories F and K shared serum panels.

Figure 1

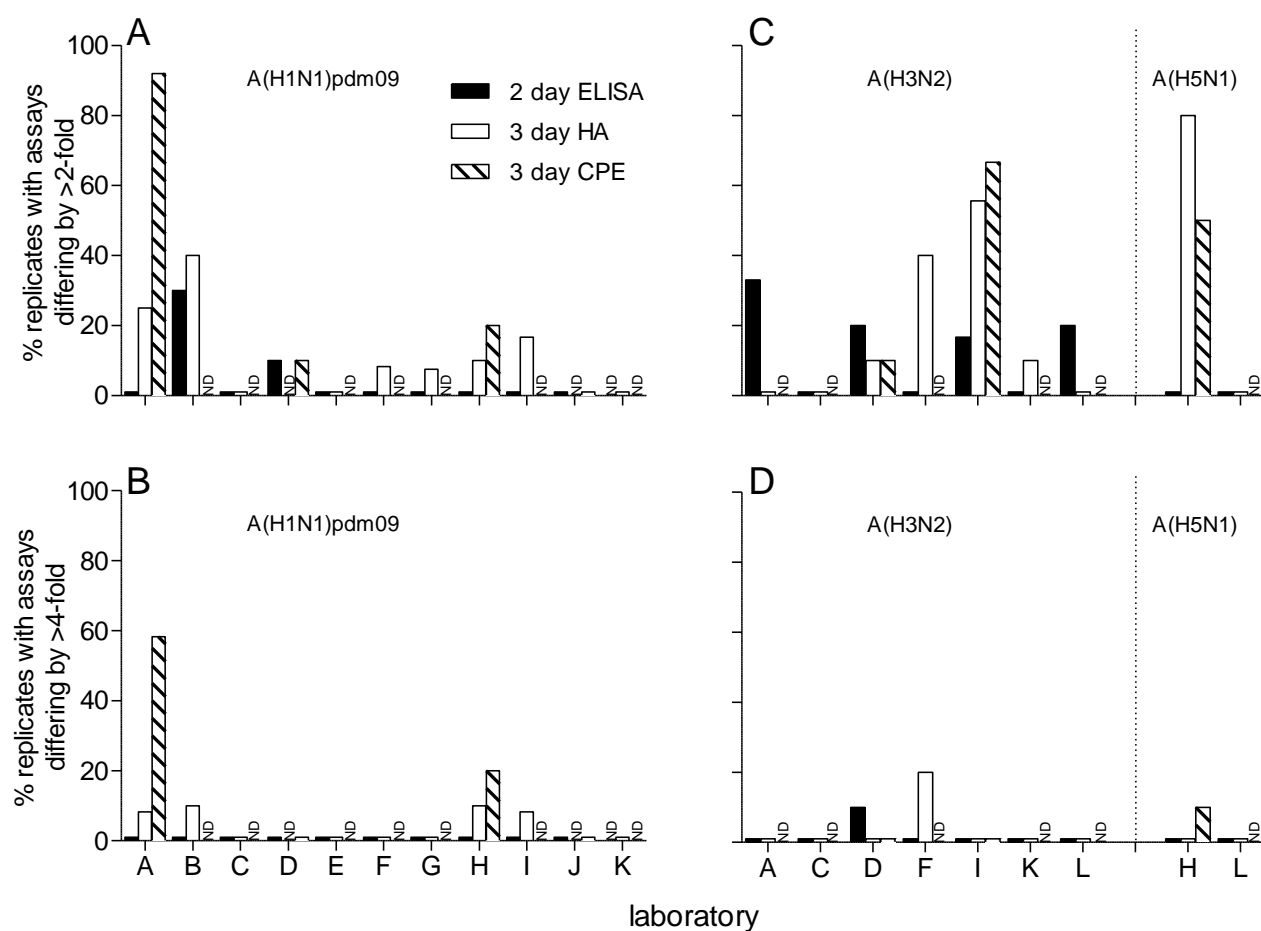


Figure 2

