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Original Article

Antibody responses to influenza viruses in paediatric patients and their contacts at the onset of the 2009 pandemic in Mexico

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Abstract

Introduction: On April 2009, the Mexican Ministry of Health received notification of cases of severe pneumonia mostly affecting young healthy people; this was the beginning of the first influenza pandemic of the 21st century. The nature of the immune response to the influenza A(H1N1)2009 pandemic strain in Mexico at the beginning of the pandemic outbreak has not been completely defined. We describe the serological response to the 2009 pandemic influenza virus in paediatric patients with influenza-like illness, their household contacts (HHCs), and exposed health-care workers (HCWs) at the beginning of the pandemic outbreak in Mexico City.

Methodology: thirty pre-epidemic and 129 epidemic samples were collected and serum antibodies were measured against A(H1N1)2009 pandemic virus and two non-pandemic swine influenza viruses by an haemagglutination inhibition assay .

Results: 91% (29/32) of the convalescence samples from confirmed patients had an antibody titre ≥ 10 (GMT 25), 63% (41/65) of the HHCs (GMT 12), 41% of HCWs (GMT 6) and 13% (4/30) of pre-epidemic samples (GMT 6) for the pandemic influenza virus. Of the 32 confirmed cases, 60% had an antibody titre ≥ 40 for the pandemic strain, 53% for the A/swine/Iowa(H1N1) virus (GMT 62) and 43% for the A/swine/Texas(H3N2) virus (GMT 66).

Conclusion: The antibody response to 2009 pandemic influenza virus was widespread in convalescence samples from patients with confirmed pandemic influenza infection but the GMT was below the protective titre. There was no evidence that antibodies to the swine influenza viruses had cross-protective effect against the 2009 pandemic influenza virus.

Key words: Pandemic influenza; H1N1; antibody response; household contact; healthcare worker.

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Introduction

On April 14, 2009, the Mexican Ministry of Health received notification of cases of severe pneumonia mostly affecting young healthy people [1], this was the beginning of the first influenza pandemic of this century that was declared by the World Health Organization (WHO) on June 11, 2009. Data on the genetic composition of the virus indicated that A(H1N1) 2009 pandemic influenza virus (A(H1N1)pdm09) contained genes from avian, human and swine influenza viruses and probably resulted from the reassortment of recent North American H3N2 and H1N2 swine viruses with Eurasian avian-like swine viruses [2,3]. Information about pre-existing immunity to A(H1N1)pdm09 virus and seroconversion after exposure in different populations is available

from published studies [4,5]. Hancock *et al*. reported cross-reactive antibodies to A(H1N1)pdm09 virus, in stored serum samples from people who either were blood donors or recently seasonal influenza-vaccinated and individuals vaccinated with the 1976 swine-vaccine. Subjects born before 1930, and probably exposed to a 1918-like H1N1 virus had the highest titres against A(H1N1)pdm09 virus [6]. Ikonen *et al*. demonstrated in a collection of sera taken before 2004 in Finland, that people born before 1944 had pre-existing immunity [7]. Around 1998, a human H3N2 virus reassorted with an avian influenza virus and a common swine H1N1 virus resulting in a triple reassortant that was the precursor of the current A(H1N1)pdm09 virus [8].

In order to explore whether a group of children had had previous exposure to swine viruses and whether prior exposure to these viruses had any influence on the population exposed to the A(H1N1)pdm09 virus, the serological response against A(H1N1)pdm09 virus infection and two non-pandemic swine influenza A viruses, in a group of paediatric patients were analysed; children presented with influenza-like illness (ILI): fever ≥ 100 F° plus cough and/or sore throat, also their household contacts and health-care workers (HCW) exposed to the virus at the beginning of the pandemic outbreak at the epicentre were investigated.

Methodology

Blood samples

Thirty pre-epidemic stored samples were obtained from banked sera from the Paediatrics Hospital of the National Medical Centre "Siglo XXI" from the Mexican Social Security Institute (IMSS) in Mexico City, Mexico from December 2008 to March 2009. These samples were drawn as part of the study protocol for transplant patients and donors; three to five mL of blood from each subject were drawn by venous puncture with sterile equipment, after signing informed consent. The consent form states that samples can be preserved to perform further studies, so each sample was anonymised and stored at -70 ºC. The collection included 28 children with a median age of 9 years and two adults (organ donors, 22 and 41 years old), and was used as the control pre-epidemic group. None of the subjects were suffering an acute infectious disease.

One hundred and twenty-nine samples were taken during the epidemic waves (between April 23 and November 15, 2009) from patients, household contacts and HCWs. Written consent was obtained from children parents and adult contacts. Study approval was obtained from the IMSS through the National Commission of Scientific Research, which comprises the Scientific, Ethics, and Biosafety Committees, in accordance with Good Clinical Practice. Three to five mL of blood from each subject were drawn by venous puncture with sterile equipment, and stored using the same procedure performed for control samples. The case status definitions were: a) ILI: a fever ≥ 100 F° plus cough and/or sore throat; b) confirmed case: a patient with ILI and a positive real time PCR test (RT-PCR) for A(H1N1) 2009 pandemic influenza virus; c) negative or discarded case: a patient with ILI but negative for rapid test (QuickVue Influenza Test A+B; Quidel, San Diego, USA) [9] and RT-PCR test; d) household contact: any member who have spent at least 4 hours/day on average ±7 days from illness onset in the confirmed case, and e) exposed health-care worker (HCW): a healthcare worker in close contact (within 6 feet) with confirmed or suspected cases [10]. Data collected included general demographic information, history of seasonal influenza vaccination, number of household members, underlying diseases and respiratory symptoms. Respiratory specimens included nasopharyngeal swabs, pharyngeal swabs from non-critically ill patients, and bronchial aspirates taken by tracheal suction or bronchoscopy from patients with mechanical ventilation. The samples were processed for detection of influenza virus by real-time reverse transcriptase PCR (rRT-PCR) assays based on CDC protocols [11]. CDC personnel trained and supervised CDC/WHO protocol for Real Time PCR in Mexico-INDRE (Institute for Epidemiological Diagnosis and Reference).

Haemagglutination inhibition assay

The sera were tested for anti-influenza Abs using an HIA, according to procedures described previously [12,13] with some modifications; 3 volumes of serum were heat-inactivated at 56°C for 30 minutes, adsorbed with 2 volumes of 12.5 % kaolin solution (v/v in PBS) (Sigma-Aldrich, St. Louis, USA) and two volumes of 5% chicken red blood cells (RBC) overnight at 4 °C. After centrifugation at 3000 *g* for 10 minutes, the supernatant (a 1:10 dilution of kaolin-treated serum) was retrieved. Before each test, the virus titre was standardized to a dilution of 8 haemagglutination units (HIU)/50µL PBS pH 7.0. The HIA assay was performed using a 0.5% chicken RBC suspension (v/v in PBS), serial two-fold dilutions of serum up to the 8th well (starting at 1:10 dilution) and in U-bottom 96-well plates (Nunc, 449824, Roskilde, Denmark). Each

test included a negative control without virus. The reciprocal of the highest serum dilution that totally inhibited RBC agglutination was assigned as the HI titre. The assay was performed with the influenza A viruses: pandemic A/Mexico/4482/2009(H1N1) and two non-pandemic swine strains: A/swine/Iowa/00239/2004(H1N1), and A/swine/Texas/4199-2/98(H3N2). The strain A/Mexico/4482/2009(H1N1) is a clinical isolate obtained from a Mexican patient diagnosed with pandemic influenza during the first wave in 2009. A/Swine/Iowa/00239/2004(H1N1) and A/Swine/Texas/4199-2/98(H3N2) were chosen because of the similarity between the haemagglutinin aminoacid sequence of these strains and the pandemic H1N1 virus, the seasonal A/Brisbane/59/2007(H1N1) and A/Brisbane/10/2007(H3N2), respectively. The phylogenetic tree, together with the identity matrix (Supplementary Figure 1 and Supplementary Table 1) indicates a high degree of identity (> 0.9) between the H1N1 strains used for the study (A/Mexico/4482/2009(H1N1), A/swine/Iowa/00239/2004(H1N1)) and the reference sequences for the pandemic strains (A/California/04/2009, A/California/07/2009). The same degree of identity (0.913) is observed for the H3N2 strain used for the study (A/swine/Texas/4199-2/98) and the circulating H3N2 strain during the outbreak (A/Brisbane/10/2007). Although the virus A/Swine/Iowa/00239/2004(H1N1) is phylogenetically rather far to the seasonal strain A/Brisbane/59/2007(H1N1), analysis of haemagglutinin aminoacid sequence showed a high degree of identity (0.791), for which it was used also to measure antibodies against seasonal H1N1 virus.

Undetectable samples were defined as those having HIA titres < 10. Geometric mean titre (GMT) is the geometric mean of the positive HI titres. Titres were log-transformed to calculate the GMT and 95% confidence intervals (95% CI). Undetectable HIA titres were assigned an arbitrary value of 5 to calculate the GMT. Samples were considered seropositive with a titre of $\geq$ 10, and a titre of $\geq$ 40 were considered seroprotective [6,14].

Statistical analysis

GMT with 95% CI was calculated for each case or subject status group: confirmed cases, discarded cases, household contacts, health care workers and control groups. All subjects were also divided in six age groups. The proportion of samples with antibody titres for titres $\geq$ 10 and $\geq$ 40 were registered for all the groups. All calculations were performed for the three strains. One-way ANOVA was used to compare mean log titres between groups. Bonferroni correction was performed to compare five subgroups to test difference between them. Based on the family wise rate, significance level was established at $\leq$ 0.01.

Chi square test was used to compare difference in proportions, a p value $\leq$ 0.05 was considered significant. All analyses were performed with IBM SPSS Statistics v. 20.0.

Results

Samples from 30 pre-epidemic controls, 42 paediatric patients with ILI/pneumonia, 65 household contacts and 22 health-care workers were evaluated in this study.

In 76% of the paediatric patients (32/42), the result of the RT-PCR test confirmed A(H1N1)pdm09 virus infection (Table 1). Three of the ten samples that were negative by RT-PCR, were positive in the rapid test for influenza A. Antibody titres of 10-320 to A(H1N1)pdm09 strain were reported in 29/32

Table 1. Epidemiological and clinical data of the groups included in this study.

Group (Sample collection dates)		Total N	Median age (range)	Presentation of disease
Pre-epidemic controls (Dec 2008-March 2009)		30	9 years (1 – 41 years)	Asymptomatic
ILI*/pneumonia paediatric patients (April 23-November 15, 2009)	Confirmed cases	32	8 years (4m – 17 years)	ILI*/pneumonia
	Positive rapid test	3		
	Discarded (negative test)	7		
Household contacts (May 30-November 15, 2009)	Adults	48	35 years (18 – 74 years)	Asymptomatic
	Children	17	6 years (3m – 16 years)	Asymptomatic
Exposed health-care workers (May 30-November 15, 2009)		22	32 years (25 – 48 years)	Asymptomatic

*ILI = influenza-like illness

confirmed patients in the convalescence period (14 to 90 days after onset of illness). The negative results were from patients with altered immune status: a) one patient with severe malnutrition and congenital heart disease, b) one with systemic lupus erythematous, and c) one with chronic renal failure.

The three patients with negative RT-PCR test and a positive rapid test, had antibody titres ≥10 (GMT 17). All patients in the discarded group tested negative in the HIA (Table 2).

Nine of 32 confirmed patients died (lethality 28%). The antibody titres were not statistically different in patients with severe disease in comparison with moderate to mild disease ($p > 0.05$).

Sixty-five samples were obtained from household contacts (median 1.5 contacts per patient, range 3-9). Blood samples were taken 20-90 days after onset of illness in the patients with ILI/pneumonia. None household contacts had ILI before the onset of illness in the paediatric patient, seven subjects developed an upper respiratory tract infection during the first week after contact with a confirmed case; neither of them fulfils ILI definition criteria nor underwent confirmatory tests.

Pre-epidemic samples (December 2008-March 2009) included 28 children with a median age of 9 years, and two adults (organ donors, 22 and 41 years old). Four of the 30 samples (13%) in the control group were positive to A(H1N1)pdm09 virus, with a GMT titre of 6 (Table 2). None of the subjects had symptoms of respiratory illness. Results of Bonferroni comparisons showed statistical significant differences in the serologic response between the virological confirmed cases and subjects in the discarded group and pre-epidemic control group ($p < 0.01$). There was also a significant difference between the household contact group and the pre-epidemic control group ($p < 0.0001$). The rest of the comparisons were not statistically significant (Table 3).

Serum HIA to A(H1N1)pdm09 virus were analysed by age group in the 159 samples. Fifty six per cent (89/159) of all the samples had a detectable antibody titre ≥ 10, but only in 28% (45/159) the titre reached a seroprotective value ≥ 40, with the lower frequency for those in the 30-39 years group ($p < 0.05$) (Figure 1).

Table 2. Antibodies to A (H1N1) 2009 pandemic influenza virus in the sera of patients, controls, household contacts and exposed health-care workers (HI assay).

Group		Total N	Titre ≥ 10 n (%)	GMT** (95% confidence interval)
Pre-epidemic controls		30	4 (13%)	6 (5% – 85%)
ILI/pneumonia patients	Confirmed cases	32	29 (91%)	25 (11% – 43%)
	Positive rapid test	3	3 (100%)	17 (11% – 128%)
	Discarded (negative test)	7	0 (0%)	5 [(a)]
Household contacts		65	41 (63%)	12 (8% – 17%)
Exposed health-care workers		22	9 (41%)	6 (5% – 12%)

*ILI =influenza-like illness; **GMT = geometric mean titre; [(a)] Undetectable titres; an arbitrary value of 5 was assigned.

Table 3. Comparison of mean log antibody titres to A/Mexico/4482/2009(H1N1) between groups according to case or subject status. Post-hoc analysis.

(I) Case or subject status	(J) Case or subject status	Mean Difference (I-J)	95% Confidence Interval		p*
Confirmed cases	Only positive by rapid test	0.0638	-1.1324	1.2600	1.000
	Discarded cases	1.0638	0.2372	1.8904	0.003
	Household contact	0.2802	-0.1476	0.7080	0.636
	Health care worker	0.5863	0.0376	1.1350	0.028
	Pre-epidemic Control	0.8981	0.4139	1.3823	< 0.001
Household contact	Confirmed cases	-0.2802	-0.7080	0.1476	0.636
	Only positive by rapid test	-0.2164	-1.3863	0.9535	1.000
	Discarded cases	0.7836	-0.0045	1.5717	0.052
	Health care worker	0.3061	-0.1826	0.7947	0.758
	pre-epidemic Control	0.6212	0.2006	0.0417	< 0.001

* comparison case or subject status (I) versus case or subject status (J)

Figure 1. Proportion of samples with ≥ 40 antibody titres against influenza A (H1N1) 2009 virus from all 159 samples (pre-epidemic controls, ILI/pneumonia patients, household contacts and exposed health-care workers), according to age group.

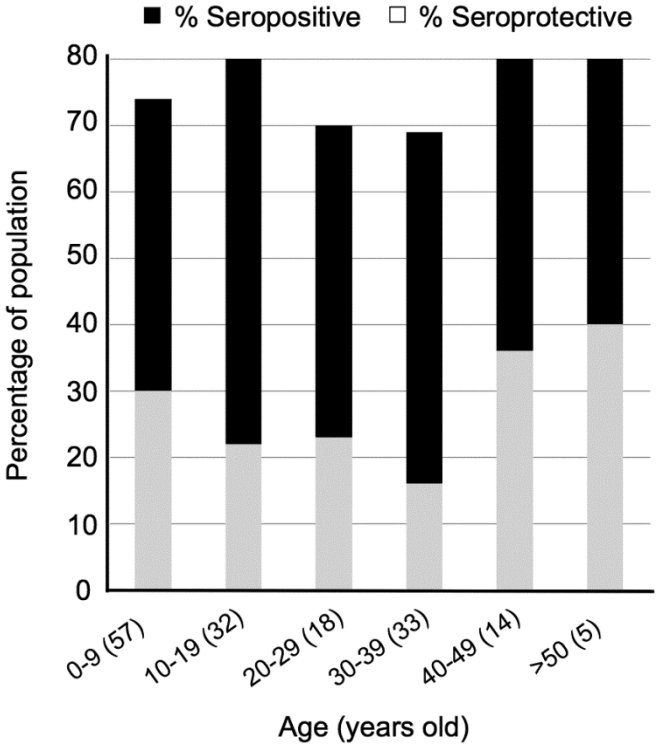

Figure 2. Comparison of GMTs to influenza A (H1N1) 2009, A/swine/Iowa/00239/2004 (H1N1) and A/swine/Texas/4199-2/98 (H3N2) viruses, in the serum of all subjects (n = 159), according to their age group.

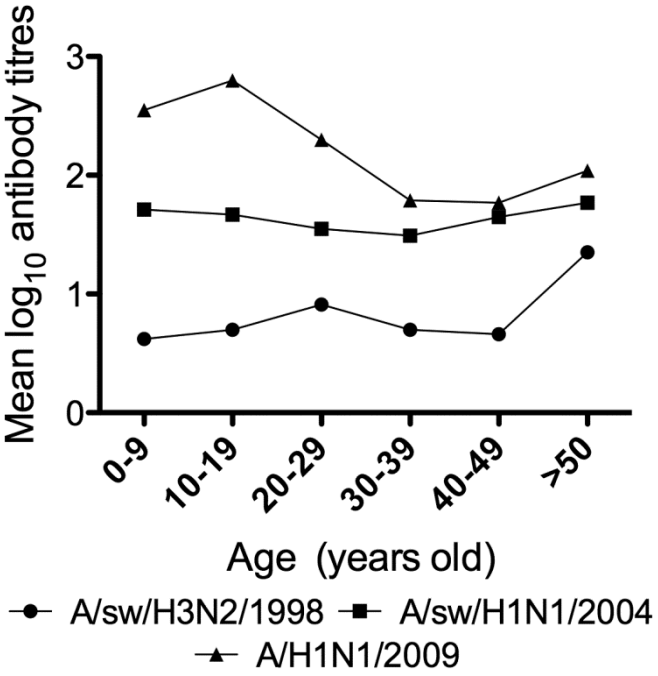

Table 4. Antibodies to influenza A/Mexico/4482/2009 (H1N1), A/swine/Iowa/00239/2004 (H1N1) and A/swine/Texas/4199-2/98 (H3N2) viruses.

		Virus strain					
		A/Mexico/4482/ 2009 (H1N1)		A/swine/Iowa/00239/2004 (H1N1)		A/swine/Texas/4199-2/98 (H3N2)	
Group		% Prot	GMT	% Prot	GMT	% Prot	GMT
Pre-epidemic controls		3%	5	67%	57	100%	176
Paediatric patients	Confirmed cases	60%	25	53%	62	43%	66
	Positive rapid test	33%	17	66%	90	33%	256
	Discarded (negative test)	0%	5	71%	76	42%	161
Household contacts		51%	12	65%	41	64%	58
Exposed health-care workers		33%	6	29%	24	59%	48
Total		45%	11	64%	45	64%	78

GMT, geometric mean titre; % Prot: Percentage of samples with an antibody titre ≥ 40

The antibody response to non-pandemic swine flu strains A/swine/Iowa/00239/2004(H1N1) and A/swine/Texas/4199-2/98(H3N2), revealed a high proportion of positive samples with a titre ≥ 40 (64%) in comparison to the antibody responses to the A/Mexico/4482/2009(H1N1) pandemic virus (45%) (Table 4). In the confirmed case group, the GMT to A/swine/Iowa (H1N1), and A/swine/Texas (H3N2) was 62 and 66 respectively, in contrast with a GMT of 25 against the A/Mexico/4482/2009(H1N1) pandemic virus. The mean antibody titres (log_{10}) were highest against the A/swine/Texas/1998(H3N2) strain, while the lowest mean titres were observed against A/Mexico/4482/2009(H1N1) pandemic influenza virus. Titres against A/Mexico/4482/2009(H1N1) were higher in the elderly group, but the difference did not reach statistical significance (Figure 2).

Discussion

The results of seroepidemiological surveys cannot predict the behaviour of the disease in exposed populations. The current study included ILI/pneumonia patients, their household contacts and exposed HCWs. The overall seroprevalence for A/Mexico/4482/2009(H1N1) pandemic virus was 45%, indicating that 55% of this exposed population remained without immunity to the pandemic virus. Even in the confirmed case group, the GMT was relatively low (25) and is not considered protective [14,15]. There is evidence that A(H1N1)pdm09 virus induces a weak host immune response [16,17], which could explain the low antibody titres. Hung *et al.* reported a similar percentage of seronegative patients, when they found that 10% of confirmed patients did not generate antibody responses to the pandemic virus [15]. Our data indicate that the presence of high titres of antibodies to the non-pandemic strains A/swine/Iowa/00239/2004(H1N1) and A/swine/Texas/4199-2/98(H3N2) did not generate protection to the A(H1N1)pdm09 virus infection, since hospitalised patients with confirmed pandemic influenza had protective antibody titres to these swine viruses.

HIA antibodies to A(H1N1)pdm09 virus were found in three patients with a negative RT-PCR result against both pandemic and swine influenza virus. These patients presented with severe lethal flu-like disease, suggesting a false negative result, this may occur due to improper collection, transport or handling. It has also been reported that a false negative result may occur if an excess of DNA/RNA template is present in the reaction, and dilutions must be performed to verify the result [11]. The other seven patients with negative rapid test and RT-PCR results had undetectable antibodies against A(H1N1)pdm09 virus.

The seroprevalence in household contacts was higher than in the HCWs (51% vs. 33%, respectively). It is common that the care of a sick child relies on one member of the family; the GMT of the household contacts was lower than the one in the confirmed group (12 vs. 25), however, none of the 65 household contacts developed ILI. Data from other studies also describes a very low attack rate [18-20].

In Singapore Chen et al. reported a 13% rate of seroconversion in community members, but for HCWs seroconversion was relatively low (7%). In this study the lower seroprevalence in HCWs compared to household contacts could be due to the HCW reduced time of exposure, and to the use of personal protective equipment [21]. In a medical centre in Taiwan, 20% of HCWs had seroprotective titres compared to 3% of the control group ($p < 0.001$) [22]. HCW will continue to be at the top of the priority list for vaccination, as it seems that, based on seroprevalence and seroprotection data, a substantial proportion of HCWs remain susceptible even after the first waves of the pandemic.

The study has several limitations. Some of the patients with underlying chronic conditions did not develop a serological response, but the number is limited and we cannot establish that this will be the case for all patients. The secondary cases did not undergo a complete epidemiological study with the confirmatory test at occurrence of respiratory symptoms. History of seasonal influenza vaccination was available only for 43 subjects, and does not include vaccination before 2008. Despite these flaws, the present data of age-group specific susceptibility could provide useful information for public health policies, to target all the populations with an increased risk of dying due to complications of pandemic 2009 influenza A infection. Our data indicates that waves of A(H1N1)pdm09 virus did not generate antibody titres considered protective, suggesting that the population is still susceptible to infection and disease. These data could be relevant to improve vaccination coverage, and target groups not considered as high-risk subjects for complications of influenza infection. In Mexico, between April 1 through December 31, 2009, the mean case fatality ratio was 0.6% for those < 18 years, 2.8% for the group of 18–49 years and 8.5% for > 50 years [23].

Conclusion

The antibody response to A(H1N1)pdm09 virus was widespread in convalescent samples from patients with confirmed cases of pandemic influenza, but the GMT was relatively low and failed to reach a protective titre. There was no evidence that antibodies to two selected non-pandemic swine influenza viruses had cross-protective effect against A(H1N1)pdm09 virus.

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Authors' contributions

Miranda-Novales G. Concept and design, analysis and interpretation of data, patients' enrolment, writing and final approval. Arriaga-Pizano L, Ferat-Osorio E. Data analysis. Herrera-Castillo C. Concept and design, patients' enrolment. Pastelin-Palacios R, Valero-Pacheco N, Pérez-Toledo M. Implementation of HIA. Antibody titre determination. Solórzano-Santos F. Review of the intellectual content. Vázquez-Rosales G. Interpretation of data. Espitia-Pinzón C. Viruses growth, implementation of HIA. Zamudio-Lugo I. Meza-Chávez A. Concept and design, volunteers' enrollment. Klenerman P. Data analysis and interpretation, review of the manuscript. Isibasi A. Writing manuscript, laboratory infrastructure. López-Macías C. Study coordinator, conception and design, data analysis, interpretation, writing and final approval.

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Supplementary Items

Supplementary Table 1. Identity matrix for the haemagglutinin aminoacid sequences of selected strains

	A/Swine/Texas/4199/1998 (H3N2)	A/Brisbane/10/2007 (H3N2)	A/California/04/2009 (H1N1)	A/California/07/2009 (H1N1)	A/Mexico/4482/2009 (H1N1)	A/swine/Iowa/00239/2004 (H1N1)	A/Brisbane/59/2007 (H1N1)
A/Swine/Texas/4199/1998 (H3N2)	---	---	---	---	---	---	---
A/Brisbane/10/2007 (H3N2)	**0.913**	---	---	---	---	---	---
A/California/04/2009 (H1N1)	0.419	0.415	---	---	---	---	---
A/California/07/2009 (H1N1)	0.419	0.415	**0.994**	---	---	---	---
A/Mexico/4482/2009 (H1N1)	0.42	0.417	**0.994**	**0.992**	---	---	---
A/swine/Iowa/00239/2004 (H1N1)	0.410	0.412	0.913	0.911	**0.911**	---	---
A/Brisbane/59/2007 (H1N1)	0.407	0.409	0.793	0.791	0.791	**0.791**	---

Supplementary figure 1. Phylogenetic tree for the haemagglutinin gene of the strains used in this study and related strains reported, as of 21 October 2014. Sequences of strains used for the present study are shown in blue. Sequences of reference strains for 2009 pandemic influenza virus are shown in red. Sequence of H1N1 and H3N2 circulating strain during the pandemic outbreak is shown in green. GenBank accession numbers are indicated for each sequence in brackets. The phylogenetic tree was generated in the MOLE-BLAST service available in http://blast.ncbi.nlm.nih.gov/moleblast/moleblast.cgi, using the following accession numbers as queries: GQ162190.1, FJ969540.1, CY039087.1, CY095675.1, CY163864.1, FJ966082.1, KM198690.1, GQ162190.1. The Fast Minimum Evolution tree method was used for the construction of the tree.