Evolution of an influenza pandemic in 13 countries from 5 continents monitored by protein microarray from neonatal screening bloodspots

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ABSTRACT

Background: Because of lack of worldwide standardization of influenza virus surveillance, comparison between countries of impact of a pandemic is challenging. For that, other approaches to allow internationally comparative serosurveys are welcome.

Objectives: Here we explore the use of neonatal screening dried blood spots to monitor the trends of the 2009 influenza A (H1N1) pdm virus by the use of a protein microarray.

Study design: We contacted colleagues from neonatal screening laboratories and asked for their willingness to participate in a study by testing anonymized neonatal screening bloodspots collected during the course of the pandemic. In total, 7749 dried blood spots from 13 countries in 5 continents where analyzed by using a protein microarray containing HA1 recombinant proteins derived from pandemic influenza A (H1N1) 2009 as well as seasonal influenza viruses.

Results: Results confirm the early start of the pandemic with extensive circulation in the US and Canada, when circulation of the new virus was limited in other parts of the world. The data collected from sites in Mexico suggested limited circulation of the virus during the early pandemic phase in this country. In contrast and to our surprise, an increase in seroprevalence early in 2009 was noted in the dataset from Argentina, suggestive of much more widespread circulation of the novel virus in this country than in Mexico.

Conclusions: We conclude that this uniform serological testing of samples from a highly standardized screening system offers an interesting opportunity for monitoring population level attack rates of widespread diseases outbreaks and pandemics.

1. Background

In April 2009, a novel influenza A (H1N1) pdm virus emerged from Mexico, and quickly spread all over the world, causing a pandemic. It was estimated that the pandemic affected tens of millions of persons, but such estimates are difficult to obtain [1]. Surveillance across countries is not standardized, making direct comparison between countries of impacts of a pandemic based on case detection rates very difficult [2]. Population-based serological surveys can be helpful to get a better picture of the attack rate of an outbreak or widespread epidemic, and comparative analysis of age-structured seroprevalence data with notifications based on clinical parameters has helped determine population impact across age groups. To set up an active serological surveillance for influenza virus, serum samples should be collected on a regular basis, but this is not done routinely in most countries. As a result, the first population-based serological studies were reported eight-to-nine months after the initial start of the pandemic [2], when testing residual sera from diagnostic laboratories provided valuable information [3,4].

A second challenge when performing serological studies is the variability between laboratories, when using the gold standard test methods that employ biological reagents such as animal red blood cells (in hemagglutination inhibition assays (HAI)) or living cells (microneutralization test (MNT)) [5]. A review of studies from individual countries suggested differences in the proportion of persons with influenza A (H1N1) pdm cross-reactive antibodies prior to the pandemic in different countries, but it is difficult to disentangle test variation from true differences. Evaluation of such studies in the wake of the 2009 pandemic concluded that there is a need for more standardized approaches to serosurveys, including the laboratory testing, to determine the real impact of the pandemic more easily [6–8].

Dried blood spot (DBS) cards have been used for decades in neonatal screening [9]. The highly standardized, easy way of sampling and the stability of the DBS, once dried, are major advantages of this screening sample method [10]. The use of DBS for diagnostics is thus expanding, with applications based on detection of viral genome, antibodies and other molecules such as antiviral drugs [11,12].

2. Objectives

Here, we explored the possible use of routinely collected dried blood spot cards from neonatal screening programs for serological surveillance of influenza virus by the use of protein microarray [13].

3. Study design

Following notification of the emergence of a novel influenza virus strain in humans, we contacted colleagues from neonatal screening laboratories and asked for their willingness to participate in a study to monitor the trends of the influenza A (H1N1) pdm virus by testing anonymized neonatal screening bloodspots. In total, 15 laboratories worldwide agreed to participate. A study protocol was drafted and each participant checked compliance against local medical ethical rules. Laboratories were located in 13 different countries from 5 different continents (Supplemental Information 1). Participating laboratories agreed to collecting 10 randomly selected anonymized DBS per week, in concordance with policies of local ethical committees. The collection period differed per country (Table 1). After collection, DBS were stored at temperature (4 °C to room temperature) and humidity controlled environment, before

Table 1
Number of collected DBS per week for each country.

Table 2
Commercially available recombinant Hemagglutinins (HA1 part) used in this study.

Code	Influenza virus strain	Manufacturer
H1–18	A/South Carolina/1/18 (H1N1)	Immune Technology Corp.
H1–33	A/WS/33 (H1N1)	Immune Technology Corp.
H1–99	A/New Caledonia/20/99 (H1N1)	Immune Technology Corp.
H1–07	A/Brisbane/59/2007 (H1N1)	Immune Technology Corp
H1–09	A/California/6/2009 (H1N1)	Immune Technology Corp.
H2–05	A/Canada/720/05 (H2N2)	Immune Technology Corp.
H3–03	A/Wyoming/3/03 (H3N2)	Immune Technology Corp.
H3–07	A/Brisbane/10/2007 (H3N2)	Immune Technology Corp.
H5–04	A/Vietnam/1194/2004 (H5N1)	Immune Technology Corp.
H5–06	A/Turkey/15/2006 (H5N1)	Genscript
H5–05	A/Indonesia/5/2005 (H5N1)	Genscript
H7–03	A/chicken/Netherlands/1/03 (H7N7)	Immune Technology Corp.
H9–99	A/guinea fowl/Hong Kong/WF10/99 (H9N2)	Immune Technology Corp.
H9–07	A/chicken/Yunnan/YA114/2007 (H9N2)	Genscript

sending them to the RIVM per courier (time for travelling ranged up to 6 days). Upon arrival at the RIVM the DBS were stored at −20 °C until further testing.

3.1. Sampling protocol

All laboratories used filter paper of comparable absorbance specifications (either Whatman 903, Ahlstrom 226 or Toyo Roshi 545) [14]. Incompletely saturated DBS were discarded. In addition, the first 24 weeks of DBS from one of the Mexican laboratories (Mexico 1, in Supplemental Information 1) were rejected, because plasma failed to elute from the filter paper and all sera were negative when tested on microarray, including seasonal influenza virus antigens used as positive controls. From week 26 onwards, this problem no longer occurred, and the signal from all the antigens (including the seasonal H1 and H3 types) were stable over time.

3.2. Control sample

A positive control was made by mixing a human reference serum positive for influenza A (H1N1) pdm antibodies (NIBSC, UK) [15] with 50% packed human red blood cells, followed by spotting on filter paper (Whatman 903 filter paper). After drying, blood spots of 1/8 in. (containing approximately 1.54 μl (±0.17 μl) of serum [14]) were punched out of the filter paper and stored at −20 °C until further use. One DBS of this positive control was tested on each tested slide, and was used to correct for test-to-test variation and for consistency control of assay performance throughout the study period.

### 3.3. Preparation and analysis of protein microarrays

The analyses of the protein microarray for the detection of antibodies to the HA1 part of influenza virus hemagglutinin have been described before [9]. For this study we used Oncyte AVID 64-pad nitrocellulose slides (Grace bio-Labs, Bend, USA). On each 64-pad, 14 different commercially available recombinant hemagglutinin (HA1-part) proteins (Table 2) were spotted in triplo by the use of a non-contact spotting machine (Piezorray, Perkin Elmer). Each DBS was tested in one dilution on a single 64-pad. Before analysis, DBS were incubated in 40-μl PBS containing 5% Surfact-Amps at room temperature to release serum from DBS. After one hour, 80 μl of Blotto-blockingbuffer containing 5% Surfact-Amps (Thermo Fisher Scientific Inc., Rockford, USA) was added to the DBS for a final test dilution of approximately 1 in 80. Slides were placed into a 64-well incubation chamber for analysis and incubated with

Blotto-blockingbuffer. After one hour at 37 °C, slides were washed by the use of an automated microplate washer (Biotek, Winooski, USA) and incubated with 70 μl of eluted serum. After one hour at 37 °C, slides were washed and incubated with 70 μl of Blotto-blockingbuffer containing 5% Surfact-Amps and Dylight 649-conjugated goat anti-human IgG, fc-fragment specific (Jackson ImmunoResearch), diluted 1 in 1300. After one hour at 37 °C, slides were washed, including one extra washing step with water, after which the slides were dried.

After analysis, the protein microarray slides were scanned by a ScanArray scanner and spot intensities were quantified using the ScanArray Express software (PerkinElmer, Waltham, USA). To determine a proper cut-off, we used DBS available from Canada and Sweden from before April 2009. The cut-off was calculated by the mean fluorescence plus 3 times the standard deviation, resulting in a cut-off of 30,701 (fluorescence value) for antigen H1–09 (Fig. 1). Based on this, the smoothed graphical representation of the data was obtained using a generalized additive model (GAM) [16,17]. The observations were modeled via the fraction of positive bloodspots in a week by a logistic regression model, with a smooth
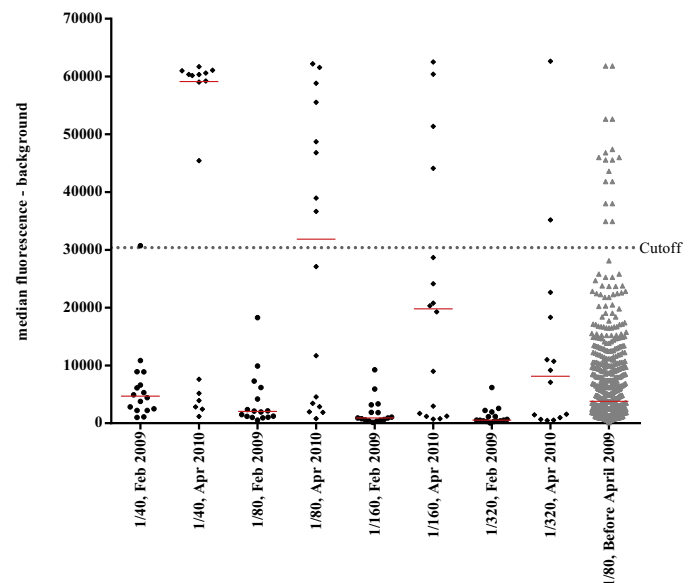
Fig. 1. Results of HA1 antibody testing of serially diluted serum eluted from DBS collected before (DBS taken on February 16, 2009) or after the pandemic (DBS taken on April 27, 2010). Red lines indicate medians. Grey dots (on the right) are DBS collected within the study before April 2009 and used to calculate the cut-off (dotted horizontal line).
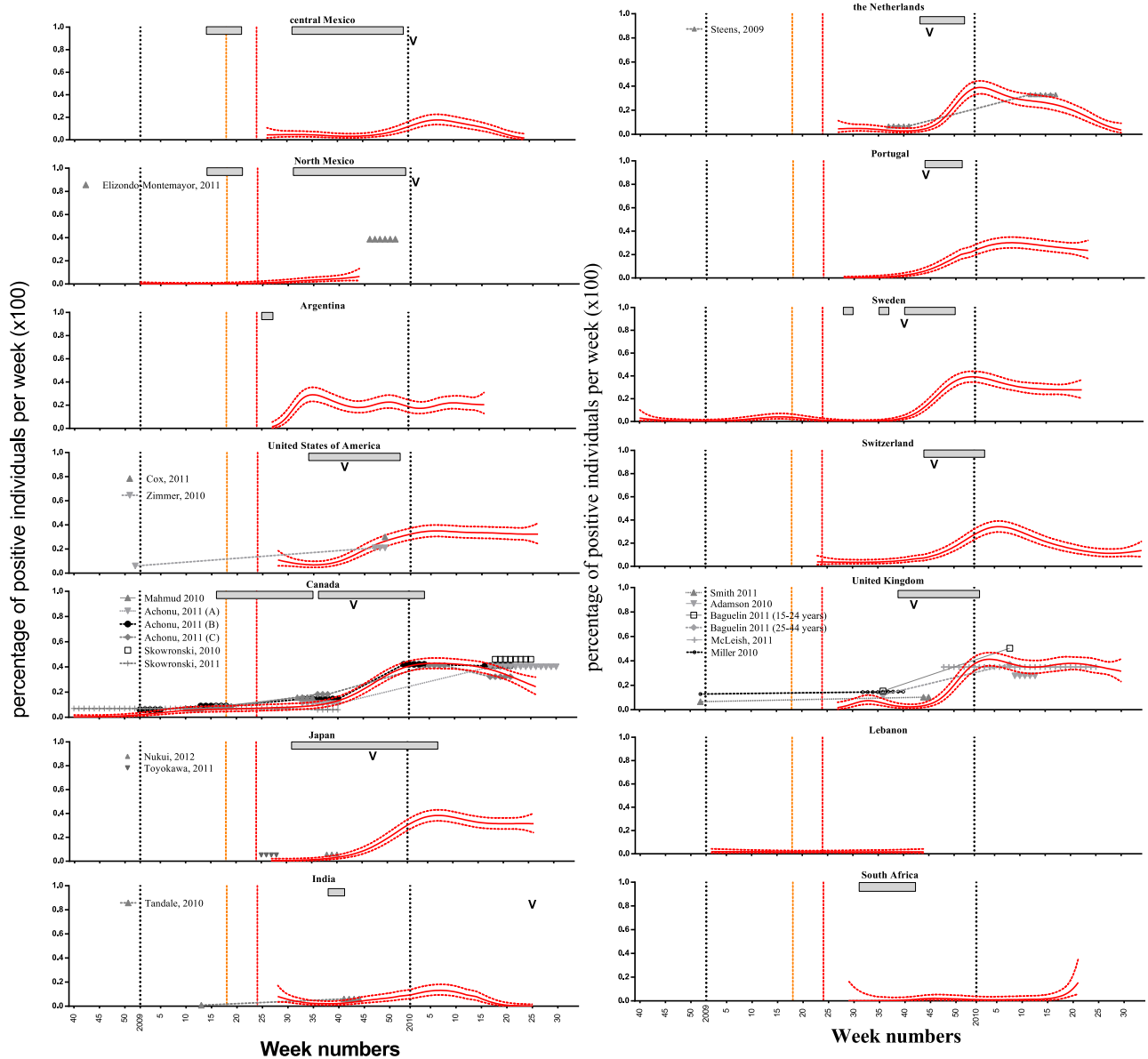
Fig. 2. Proportion of DBS (*y*-axis) per country per week (*x*-axis) testing positive for antibodies to HA1 from influenza A(H1N1) 2009. The red line is a smoothed estimation of the percentage of positive DBS per week. The dotted red lines mark the 95% confidence interval. Vertical black dotted lines mark the start of the year 2009 or 2010. The vertical orange and red lines marks the declaration of phase 5 and 6 alert, respectively, by the WHO. The horizontal grey bar within each graph depicts the epidemic period of influenza A (H1N1) pdm according to the specific surveillance data from that country (see Supplemental Information 2). The black V marks the start of influenza A (H1N1) pdm vaccination within that country. Seroprevalence studies from the literature are added for comparison marking the period tested by one or a range of triangle-, square-, dot- or plus-marks. Those marks are linked to each other when more than one period was tested during that study. If a seroprevalence study is added, the name of the first author and the line and mark, which are used to depict the study in the graph, are indicated in the upper left part of the graph.

component of time (weeks). The smooth part was defined on the logit-link scale by a thin-plate regression spline formulation [18]. Estimation of the model was done directly via penalized likelihood maximization. The degree of smoothness of the spline component was not a tuning parameter for this model, but was selected by generalized cross validation [19]. All calculations were done by the use of R (version 2.14.0) and fitting of GAM models was done by using the mgcv package in R [20]. Figures were made by the use of Graph pad Prism 5.0.4.

3.4. Review of surveillance data

Information on the onset of the pandemic wave, the start of pandemic influenza vaccination, as well as seroprevalence estimates were obtained by review of country-specific surveillance

data from institutional websites and publications, for all countries except Lebanon. This information is available as Supplemental Information and summarized in Fig. 2.

4. Results

All data points for antigen H1−09 were collected for individual countries per week, and percentages of positives were plotted after smoothing to reduce week to week variation caused by the small number of weekly samples per site. A first comparison of pre- and post-pandemic DBS from the Netherlands showed low background values within samples from non-exposed persons, and good discrimination with reactivity measured in population samples collected after the pandemic was found in a final serum dilution of 80, which was used throughout this study (Fig. 1).

Table 3
Overview of seroprevalence per time period detected in this study.

Country	Pre-pandemic, before april 2009	First detections, week 13–24	Pandemic, week 24 until december 2009	December 2009 and onwards
Central Mexico	N.T.	N.T.	10/226 (4%)	35/277 (13%)
North Mexico	0/129 (0%)	1/107 (1%)	5/196 (3%)	N.T.
Argentina	N.T.	N.T.	52/209 (25%)	55/203 (27%)
United States of America	N.T.	N.T.	32/210 (15%)	152/310 (49%)
Canada	14/477 (3%)	9/128 (7%)	36/239 (15%)	198/310 (64%)
Japan	N.T.	N.T.	10/220 (5%)	148/310 (48%)
India	N.T.	N.T.	10/199 (5%)	21/275 (8%)
The Netherlands	N.T.	N.T.	9/218 (4%)	119/341 (35%)
Portugal	N.T.	N.T.	5/209 (2%)	97/270 (36%)
Sweden	3/256 (1%)	3/120 (3%)	18/230 (8%)	126/262 (48%)
Switzerland	N.T.	0/20 (0%)	12/230 (5%)	107/387 (28%)
United Kingdom	N.T.	N.T.	10/220 (5%)	192/348 (55%)
Lebanon	2/106 (2%)	2/87 (2%)	2/144 (1%)	N.T.
South Africa	N.T.	N.T.	2/145 (1%)	3/131 (2%)
Total	19/968 (2%)	15/462 (3%)	213/2895 (7%)	1253/3424 (37%)

4.1. Pre-pandemic period, data collected before April 2009 (week 13)

Four countries (the site in Northern Mexico, Canada, Sweden and Lebanon) started collection of DBS early on, and therefore had data on reactivity of the H1 2009HA1 antigen for this time period. The number of samples testing above cut-off for this time period was 2% (Table 3). There was influenza virus activity in the northern hemisphere during this period, but related to seasonal influenza virus A (H1 and H3) and B activity. The Swedish influenza virus season reached highest incidences from week 51 (2008) to week 10 (2009) and in Canada there was a seasonal peak starting from the end of 2008 and reached highest incidence at week 9 and 10 of 2009. The end of the seasonal peak within Canada coincided with the first detections of influenza A (H1N1) pdm virus (Supplemental Information 2).

4.2. First detections, week 13–14

This period comprise the first detections of a novel influenza virus in April 2009 until the declaration of the pandemic phase 6 by the WHO (week 24). The same four countries had data for this second study period. In Mexico, Lebanon, and Sweden, the proportion positive samples remained within the 0–3% range, although visually a slight and temporary elevation was noted in the data from Sweden. The data from Canada suggested more widespread infections, reaching on average 7% seropositivity (Table 3). When reviewing data from influenza virus surveillance systems, increased activity was notified from April onwards in Mexico and Canada (depicted as grey bars above trend lines in Fig. 2, and Supplemental Information 2).

4.3. Pandemic period, from WHO phase 6 to December 2009 (week 25–48)

For this period, data were available for all participating countries. This time period was chosen because vaccination for the pandemic strain became available only towards the end of this time period, and therefore the data mostly reflects antibodies produced through natural infections. Here, the difference in onset of the pandemic is clearly seen, with increased seroprevalence in Argentina, Canada and the US. The comparative study suggests limited circulation of pandemic influenza during this study period in all other countries, including Mexico.

4.4. Pandemic period from December onward

During this period, seroprevalence data reflect a combination of natural immunity and vaccine-induced antibodies. Where available, we listed the starting dates of vaccination in Fig. 2. As our data were anonymized, we were not able to stratify samples according to vaccination history. The data suggested very limited- if any- circulation of the virus in South Africa, but clear increase in seroprevalence in all other countries for which data was available for this time period.

4.5. Comparison with published seroprevalence data

The measurements from our study were compared with published literature for the participating countries where available (Supplemental Information 3), and plotted in Fig. 2. Overall good agreement between studies was seen despite the use of different assays, except for two estimates, which were from North Mexico, and a prepandemic estimate for the UK (Fig. 2 and Supplemental Information 3). However, data from North Mexico do not overlap in sampling period, and, the pre-peak within the UK was seen mainly in England, while DBS from Scotland were tested within this study.

5. Discussion

In this large-scale study, we present data from a comparative serological study using DBS from ongoing neonatal screening programs in 13 countries, to compare the evolution of the 2009 influenza A(H1N1) pandemic using a standardized serological technique. Our study confirms the early start with extensive circulation in the US and Canada in spring of 2009, when circulation of the new virus was limited in other parts of the world until the pandemic wave towards the end of the year was observed. The study also showed some interesting differences. Data collected from sites in Mexico suggested limited circulation of the virus during the early pandemic phase in this country, which is in contrast with the hypothesis that the pandemic originated there. In contrast to the findings for Mexico and to our surprise, an increase in seroprevalence early in 2009 was noted in the dataset from Argentina, suggestive of much more widespread circulation of the novel virus in this country compared to Mexico. The newborn screening quality assurance program [14] used in this study is very standardized and used within 448 newborn screening laboratories in 61 counties (numbers for 2009). This screening program is not biased by active case finding or media attention, and the comparison of outcomes of our study with those from published serosurveys showed excellent agreement (Fig. 2). Therefore, we consider this result reliable. A question is how to explain this observation. The discrepancy between seroprevalence and presumed widespread cases cannot be explained by sampling, as two of the sites participating in our study were located in central Mexico. Alternatively, the number of cases may not have been high

enough to cause a detectable increase in seropositivity. In the Netherlands, active case finding caused a pre-peak, which was not reflected in ILI sentinel detections or this study. The same might have happened in Mexico, suggesting that impact of the epidemic in Mexico was overestimated by active case finding. Initial cases diagnosed with influenza A(H1N1) 2009 were traced to Mexico as the most likely region where infection was acquired, and reports of widespread disease suggested a large outbreak. The metropole Mexico city could have acted as a hub for dispersal of influenza viruses from imported cases, similar to what happened in the US and UK.

The impact of the pandemic was not observed everywhere during this study period. In India only a small rise in positive individuals from week 2 to 12 in 2010 was observed. The number of laboratory confirmed patients reportedly increased in the months September and October 2009 in the Andhra Pradesh province (Supplemental Information 2), but this was the only participating country that does not have a clear seasonal pattern of influenza virus [21]. During the 2009 season within South Africa, seasonal H3 and (H1N1) pdm influenza virus were both detected. Unfortunately, collecting DBS in South Africa stopped just before the second wave during the 2010 season.

While the approach presented here could be an interesting addition to case based or virological surveillance, there are disadvantages. During primary infections the three Ig classes can be detected within 10–14 days, whereas IgG level peaks 4–6 weeks after infection [22]. For that reason, serological studies are delayed in detection of infection compared to virus detection methods. Within this study, maternal IgG antibodies are measured. The antibody repertoire of the child in the first 3–12 months is dependent on the mother's accumulated immunological experience [23,24]. Thus, the antibodies detected through our approach result from the mother's history of influenza virus infections and vaccinations. Within this study, another delay comes from the time between taking DBS and birth of the infant. In the Netherlands, neonatal screening blood samples are taken 72–168 h after birth.

Because of increased morbidity and mortality from influenza virus infection, vaccination of pregnant women is recommended, but the policy for influenza virus vaccination differs per country [25]. During the pandemic, pregnant women were listed among the group at risk for severe influenza, and the numbers of infected persons might be overestimated by our approach. We were not able to find weekly estimates of the vaccination rate for pregnant women in the different countries, and the steep increases in seroprevalence in countries with active vaccination most likely reflects vaccine induced antibodies, although vaccination coincided with the onset of the pandemic wave in some countries.

Age dependent prevalence of antibodies to the influenza A (H1N1) pdm virus has been described by several publications, though findings differ among the published studies for the age group presented in this study. Some studies show higher incidence of cross reactive antibodies within the age group of approximately 18–29 years old [4,26,27], whereas other studies did not show this difference [3,28]. Although we cannot completely rule out biases due to differences between countries in average childbearing age, we found a remarkable agreement with results from published serosurveys from individual countries at specific time points, as indicated in Fig. 2. Therefore, we do not expect major influence of the sampling on the study outcomes.

We cannot rule out differences in assay performance for samples from different countries. Because of low volumes of serum per individual, it was not possible to do more tests on the DBS, such as an elution control. Nevertheless, we did test median signals for the seasonal antigens H1 and H3 that looked stable over time. DBS were stored at −20 °C for a long period before testing. Because of lack of good reference material early in 2009 to test stability during

storage it was not tested whether this might have influenced the height of the signal. However, stability of DBS is normally very good especially at −20 °C [12].

In conclusion, this standardized serological testing of samples from a highly standardized screening system offers an interesting opportunity for monitoring population level attack rates of widespread diseases outbreaks and pandemics.

Competing interest:

The authors declare no competing interests or conflict of interest.

Ethical approval:

Each participant checked compliance against local medical ethical rules. For the DBS from Japan, ethical approval was given by the Institutional Review Board of Sapporo City Institute of Public Health Judgment's (reference number 09-010). NYS DOH Institutional Review board approval was obtained for the DBS from the United States of America (protocol number #09-045).

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jcv.2014.06.020.

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