Vaccine 38 (2020) 1933–1942

Contents lists available at ScienceDirect

Vaccine

journal homepage: www.elsevier.com/locate/vaccine

Pandemic influenza A(H1N1pdm09) vaccine induced high levels of influenza-specific IgG and IgM antibodies as analyzed by enzyme immunoassay and dual-mode multiplex microarray immunoassay methods

Anna Kazakova [a], Laura Kakkola [a], Thedi Ziegler [b], Ritva Syrjänen [c], Henna Päkkilä [d], Matti Waris [a,e], Tero Soukka [d], Ilkka Julkunen [a,e,*]

[a] Institute of Biomedicine/Virology, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland
[b] Research Center for Child Psychiatry, University of Turku, Itäinen Pitkäkatu 1, 20520 Turku, Finland
[c] National Institute for Health and Welfare, Mannerheimintie 166, 00300 Helsinki, Finland
[d] Department of Biotechnology, University of Turku, Kiinamyllynkatu 10, 20520 Turku, Finland
[e] Turku University Hospital, Clinical Microbiology, Kiinamyllynkatu 10, 20520 Turku, Finland

ARTICLE INFO

Article history:
Received 3 September 2019
Received in revised form 3 January 2020
Accepted 7 January 2020
Available online 24 January 2020

Keywords:
Antibodies
Influenza A
Pandemic
Vaccine
Immunoassay
Multiplex

ABSTRACT

Influenza A viruses continue to circulate throughout the world as yearly epidemics or occasional pandemics. Influenza infections can be prevented by seasonal multivalent or monovalent pandemic vaccines. In the present study, we describe a novel multiplex microarray immunoassay (MAIA) for simultaneous measurement of virus-specific IgG and IgM antibodies using Pandemrix-vaccinated adult sera collected at day 0 and 28 and 180 days after vaccination as the study material. MAIA showed excellent correlation with a conventional enzyme immunoassay (EIA) in both IgG and IgM anti-influenza A antibodies and good correlation with hemagglutination inhibition (HI) test. Pandemrix vaccine induced 5–30 fold increases in anti-H1N1pdm09 influenza antibodies as measured by HI, EIA or MAIA. A clear increase in virus-specific IgG antibodies was found in 93–97% of vaccinees by MAIA and EIA. Virus-specific IgM antibodies were found in 90–92% of vaccinees by MAIA and EIA, respectively and IgM antibodies persisted for up to 6 months after vaccination in 55–62% of the vaccinees. Pandemic influenza vaccine induced strong anti-influenza A IgG and IgM responses that persisted several months after vaccination. MAIA was demonstrated to be an excellent method for simultaneous measurement of antiviral IgG and IgM antibodies against multiple virus antigens. Thus the method is well suitable for large scale epidemiological and vaccine immunity studies.

1. Introduction

Past influenza A virus pandemics have shown that new reassortant viruses have the potential to spread rapidly throughout the world leading to significant morbidity and mortality in humans. The latest influenza pandemic in 2009 was caused by a novel swine-origin reassortant H1N1pdm09 influenza A virus with gene segments originating from avian, human and swine influenza A viruses [1]. The possibility of avian or other animal-origin influenza A viruses to infect and spread among humans has been identified as a potential global threat that could lead to an even more severe pandemic than the previous ones. The genetic determinants responsible for the avian-to-human transmission of influenza A viruses are still partly undetermined and the ability of different strains to infect humans is not fully understood. However, there is serological evidence for bird-to-human transmission of influenza A viruses [2–5]. This highlights the need to further develop influenza surveillance systems in animals and humans [6] as well as to conduct serological surveys to monitor population immunity to various influenza types and subtypes.

Globally circulating human influenza A and B virus strains are continuously monitored and recommendations for the composition of influenza strains to be included in seasonal vaccines are updated by the World Health Organization (WHO) expert group twice a year. If novel reassortant influenza viruses are found in humans they are often used as basis for the development of

* Corresponding author at: Institute of Biomedicine, University of Turku, Medisiina D, Floor D7, Unit 7A, Kiinamyllynkatu 10, 20520 Turku, Finland.
E-mail address: ilkka.julkunen@utu.fi (I. Julkunen).

https://doi.org/10.1016/j.vaccine.2020.01.022
0264-410X/

pre-pandemic or pandemic (monocomponent) vaccines. The assessment of vaccine immunogenicity and efficacy is essential for successful vaccination campaigns [7]. Vaccines are typically developed and evaluated based on their ability to induce vaccine antigen-specific antibody responses [8]. Demonstrating the presence of antibodies to specific influenza antigens and strains is of great importance since antibodies play a prominent role in the protection against a given influenza virus strain.

The haemagglutination inhibition (HI) assay is the most commonly used method for measuring antibody levels against influenza viruses [9–11]. Anti-influenza antibodies detected by the HI assay have been shown to correlate well with protective immunity [12,13]. Another commonly used method to detect antibody responses against microbial pathogens and vaccine antigens is an enzyme immunoassay (EIA). However, there are several limitations in both HI and EIA methods. The limiting factors of these assays are that they are labor- and time-intensive and especially the HI test requires relatively large sample volumes [14,15]. Conventional EIA allows analysis of different antibody classes, however each immunoglobulin class has to be analyzed separately. HI assay has a strong limitation in analysing currently circulating influenza A (H3N2) viruses. Recent changes in the receptor binding characteristics of seasonal A(H3N2) viruses led to poor agglutination of red blood cells [16]. Therefore, inability of contemporary H3N2 viruses to be analysed by the HI assay requires the development of alternative methods.

In order to better facilitate influenza surveillance and the rapid assessment and development of vaccines, the limiting features of traditional serological assays and enzyme immunoassays have to be overcome. Modern multiplex techniques provide a great opportunity for a more broad-spectrum characterization of humoral immunity induced by vaccines and natural infections. Multiplex technology emerged about 20 years ago, first in the field of genomics and it was later widely used in proteomics, oncology, immunology, and infectious disease research [17–22]. Emerging multiplex techniques allow researchers to examine vaccine responses with greater throughput and less time [15,23,24]. Recently, multiplex protein microarray assays for influenza virus serology have been developed and the assays have shown a great potential in studies of humoral immune responses to influenza infection and influenza vaccines [25–27]. Influenza hemagglutinin antigen-based microarrays have shown to be a valuable tool in studies of specificity, cross-reactivity and cross-protection of hemagglutinin-specific antibodies [28–30]. A high density hemagglutinin protein microarray consisting of 127 different hemagglutinin antigens from 60 viruses demonstrated a high-throughput measurement of breadth of antibody diversity induced by vaccination and influenza infection [31]. Of interest is the glycan microarray technology which enables the detection of the specificity of influenza virus strains for different types of glycan structures [32]. The technology allows the analysis the human receptor specificity of avian influenza virus strains.

Here we describe the development, validation, and implementation of an in-house multiplex microarray immunoassay which enables simultaneous quantitative detection of IgM and IgG antibodies against multiple vaccine or viral antigens. In the present study, we analyzed serum specimens collected from 60 individuals before and after vaccination with pandemic influenza vaccine in 2010 in Finland. We analyzed vaccine-induced humoral immune responses and compared antibody responses determined by HI test, EIA and microarray immunoassay. We sought to determine whether the microarray immunoassay could be used instead of other more labor-intensive tests to measure influenza vaccine-induced antibody responses.

2. Materials and methods

2.1. Serum samples

A cohort of adults without any immunological disorders was recruited to a clinical and serological follow-up study on a voluntary basis between December 2009 and September 2010 in Tampere, Finland. Vaccinees received one intramuscular dose of Pandemrix™ (GlaxoSmithKlein, Rixenart, Belgium) vaccine in connection with the national pandemic vaccination campaign that was carried out in Finland in 2009 and 2010. The vaccine contained inactivated, split influenza virus propagated in eggs and an oil-in-water adjuvant AS03 [33]. The study protocol and the effectiveness of the vaccine have been previously published [34]. All participants gave their written informed consent before enrolment in the study. Serum samples were collected before the vaccination (day -3 - 0) and on an average of four weeks (day 28) and 6 months (day 180) after the vaccination. We analyzed altogether 180 serum samples from 60 individuals. The mean age of the vaccinees was 35 (range 18–65 years, median age 23 years) and 73% were females and 27% males.

2.2. Antigens and controls

H1N1pdm09 (A/California/07/2009 strain; provided by National Institute for Health and Welfare, THL, Finland) vaccine virus was propagated in chicken eggs and the virus was utilized as an antigen in the HI test. Pandemrix split whole virus vaccine (A/California/07/2009 strain; GlaxoSmithKline Biologicals) was concentrated with Amicon 10 K filter centrifugal tubes (Merck Millipore) and then the H1N1pdm09 vaccine antigen (ag) was used as a source of capture antigen in EIA and MAIA. The microarray included also partially purified influenza B virus (IBV) Yamagata strain (B/Finland/58/2011; provided by THL) and four control antigens. Purified human IgM (hIgM) was used as a positive control for the anti-hIgM coated blue-emitting UCNPs (upconverting nanophosphors; Tm-UCNP-anti-hIgM). Purified human IgG (hIgG) (Sigma-Aldrich, St. Louis, MO) was used as a positive control for anti-hIgG coated green-emitting UCNPs (Er-UCNP-anti-hIgG). UCNP labeling techniques and IgG/IgM specificity of the assay have been described before [35]. Rabbit anti-hIgG (Thermo Scientific, Rockford, IL, USA) was also printed in the microarray as a positive control for the assay. Human serum albumin (HSA), (Sigma-Aldrich, St.Louis, MO), was used as a negative control to determine the extent of non-specific binding in the assay. Capture antigens and antibodies were biotinylated and immobilized onto streptavidin-coated plates as described previously [36]. The printing concentration for the spots with biotinylated whole-virus IBV Yamagata antigen and biotinylated HSA (human serum albumin) were 400μg/ml. The protein concentration of the H1N1pdm09 vaccine antigen was 200μg/ml. The antigen concentrations of the hIgG and hIgM antibody controls were 50μg/ml and 150μg/ml for anti-hIgG control.

2.3. Serologic assays

Serum samples were analyzed by three serologic assays to determine anti- H1N1pdm09 ag antibody endpoint titers (all assays) and relative antibody units (EIA and MAIA).

Serum specimens were analyzed by the HI test using the A/California/07/2009 vaccine virus. The HI test was performed according to WHO guidelines [37] using 0.5%/vol turkey erythrocytes. For statistical analyses, serum specimens with HI titers < 10 were assigned a titer value of 5.

All serum samples from one individual were analyzed simultaneously for the presence of anti-H1N1pdm09 vaccine ag IgM and

IgG antibodies. Endpoint titers were determined by enzyme immunoassay (EIA) and microarray immunoassay (MAIA) using a series of serum dilutions. All serum samples were tested in duplicates and each plate included negative (denoted as 0 IgM/IgG units) and positive (denoted as 100 IgM/IgG units) control samples. IgM negative and positive controls consisted of seronegative and seropositive adult serum samples, taken before and after vaccination, respectively. IgG negative control sample consisted of a pool of seronegative child sera. The positive control consisted of a pool of highly positive adult sera, taken 3 weeks after the vaccination.

EIA was performed with H1N1pdm09 vaccine ag essentially as described previously [38]. The antigen was dissolved in phosphate-buffered saline (PBS), pH 7.2 at a concentration of 1.25 μg/ml and adsorbed onto the wells of polystyrene microtiter plates (Combiplate, 96-well format, Thermo Scientific, USA) in a volume of 100 μl/well. For the determination of vaccine-induced IgM antibodies serum samples were tested at five dilutions: 1/100, 1/300, 1/1000, 1/3000 and 1/10000. For the determination of vaccine-induced IgG antibodies samples were tested at dilutions of 1/1000, 1/3000, 1/10000, 1/30000 and 1/100000. For statistical analyses (geometric mean titers, GMT), serum specimens with IgM EIA titers < 100 and IgG EIA titers < 1000 were assigned a titer value of 50 and 500, respectively.

MAIA was performed using microarrays spotted on the bottom of microtiter plate wells essentially as described previously [36] with the difference that two types of detection antibody conjugates, Er-UCNP-anti-hIgG and Tm-UCNP-anti-hIgM were added into the wells simultaneously [35]. Two virus antigens (H1N1pdm09 vaccine ag and IBV Yamagata virus) and four control antigens (HSA, hIgG, hIgM, anti-hIgG) were printed in duplicates forming a 4x3 format in a microarray-in-well. The analyzed serum samples were diluted at 1/100, 1/300, 1/1000, 1/3000 and 1/10000 into the assay buffer. The upconversion luminescence (UCL) of the bound antibody conjugates was imaged both at the green 550 nm and blue 470 nm channels with an anti-Stokes photoluminescence imager to produce two images from each well. Average UCL signals from the individual microarray spots were obtained using ImageJ software, version 1.43n. Specific signals of the antigen spots were calculated by subtracting the assay HSA background from the mean signal of the antigen spots. For statistical analyses (GMT calculations), serum specimens with IgM and IgG MAIA titers < 100 were assigned a titer value of 50.

2.4. Statistical analysis

IgM cutoff level for endpoint titers and seropositivity was determined as the mean plus 3 SD (standard deviation) absorbance values (for EIA) or mean specific signal counts (for MAIA) of all pre-vaccination samples at 1/100 dilution that had an HI titer <10. The cutoff values were absorbance of 0.517 for EIA and the signal count value of 5075 for MAIA. We standardized the assays by converting the raw EIA absorbance data to EIA IgM and IgG units and raw microarray specific signal counts to MAIA IgM and IgG units using negative and positive control calibrator samples in each assay run. IgM seropositivity cutoff values at 1/100 serum dilution were 13 and 10 units for EIA and MAIA, respectively.

IgG cutoff level for endpoint titers and seropositivity was determined as 4 times mean absorbance values (for EIA) or mean plus 3 SD specific signal counts (for MAIA) of negative controls at 1/1000 dilution and were equal to absorbance value of 0.220 for EIA and signal count value of 2024 for MAIA. IgG seropositivity cutoff values at 1/1000 serum dilution were 5 and 10 units for EIA and MAIA, respectively.

EIA and MAIA units were calculated from the linear plot of the calibrator using Microsoft Excel version 2010 (Microsoft Corp., USA). EIA and MAIA endpoint titers were calculated with the 4 Parameter Logistic (4PL) curve using SigmaPlot 14.0 software. EIA, MAIA and HI assays were compared and correlated with

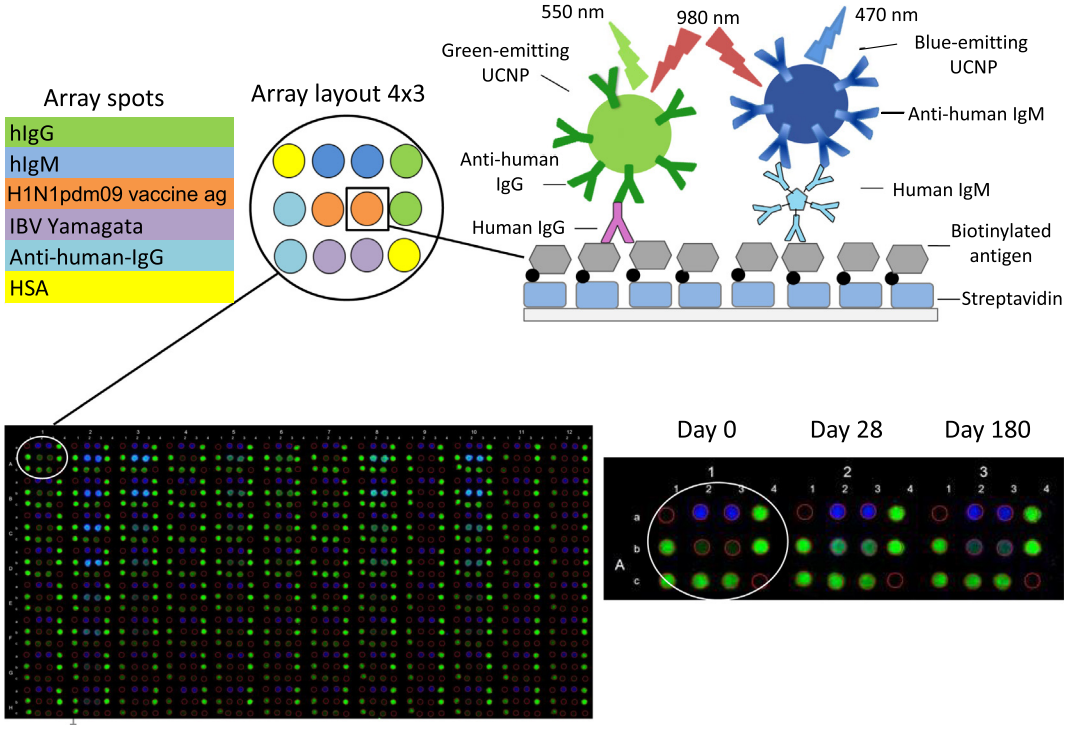

Fig. 1. Microarray immunoassay assay principle, array layout, fluorescent array-in-well images and composite fluorescent image of an example of an entire 96-well plate. The antigens (pandemic influenza A (H1N1pdm09) vaccine ag, IBV Yamagata), human serum albumin (HSA, negative control), hIgG, hIgM, and anti-hIgG (positive controls) were printed on the bottom of the wells in streptavidin-coated 96-well plate. Binding of the serum IgM and IgG antibodies to antigens and positive controls were detected by using blue-emitting anti-hIgM coated Tm-UCNP and green-emitting anti-hIgG coated Er-UNCP, respectively.

Pearson and t-tests using IBM SPSS Statistics v.22 software (IBM Corp., USA). Data on the arithmetic mean units depicted on the box plots were analyzed and graphed using Origin version 2016 (OriginLab, USA).

Geometric mean titers (GMTs) with 95% confidence intervals and theoretical "seroprotection" rates were calculated. HI titers of ≥40 were considered as seroprotective and the corresponding seroprotective titers for EIA and MAIA were determined from the correlation curves as the titer value corresponding to the HI titer of 40. Statistical differences between the groups were calculated using independent samples *t*-test (paired, two-tailed) and the statistically significant difference was set at a level of $p < 0.05$.

3. Results

3.1. Microarray immunoassay spot signals

The assay principle is shown in Fig. 1. For this study, we designed an array consisting of 12 spots. Two replicate spots of each of 2 antigens and 4 controls were printed on 4 × 3 layout on the bottom of each microtiter well. Upconversion luminescence signals from each sample well were imaged separately at blue 550 nm and green 470 nm emission channels for the detection of the bound Er-UCNP-anti-hIgG and Tm-UCNP-anti-hIgM, respectively. Serum samples collected from the same individual at day 0, day 28 and day 180 were tested simultaneously in serial dilutions in one 96-well plate. The fluorescent image of the entire 96 well-plate represents composite overlays of two separate images obtained from green and blue emission channels.

Specific signal counts were calculated for each analyte from two replicate spots in two replicate wells (Fig. 2). The blue emission from the hIgM control spots in all three serum samples taken at different time points confirmed bound Tm-UCNP-anti-hIgM (Fig. 2A). The blue emission was also observed from H1N1pdm09 vaccine ag spots from samples collected at day 28 and day 180 after the vaccination, which confirms the development of anti-vaccine IgM antibodies. The green emission was detected from hIgG, anti-hIgG, H1N1pdm09 vaccine ag and IBV Yamagata spots as a result of bound Er-UCNP-anti-hIgG (Fig. 2B). The equal signals from all three samples (days 0, 28 and 180) in IBV Yamagata spots indicated an existing immunity (IgG abs) to influenza B virus. A rise in the signal strength from H1N1pdm09 vaccine ag spots from the sample derived four weeks after influenza A vaccination, confirmed vaccine-induced immunity. We observed practically no cross-reactivity between Tm-UCNP-anti-hIgM and Er-UCNP-anti-hIgG conjugates indicating that the assay is capable of measuring IgG and IgM antibody responses separately.

3.2. Mean antibody levels and seroprevalence before and after Pandemrix vaccination

Fig. 3A shows the arithmetic mean IgM and IgG unit values, measured with H1N1pdm09 vaccine ag by EIA and MAIA in serum specimens collected before and after the vaccination. The arithmetic means of antibody values were slightly higher obtained with the EIA than those seen in the MAIA. With the exception of a few cases (possibly recently infected, n = 4 out of 60), there was no pre-existing anti-H1N1pdm09 vaccine ag IgM antibodies before the vaccination. Four weeks after the vaccination the mean IgM antibody levels (units) rose significantly 8.9–12.2-fold in EIA and MAIA assays, respectively ($p < 0.001$). As expected, the mean IgM antibody levels decreased by 38–43% by day 180 ($p < 0.001$). Pre-vaccination IgG antibodies were at levels 30 and 18 unit values by EIA and MAIA, respectively. The vaccine-induced immune responses between the pre-vaccine and 28-day post-vaccine serum

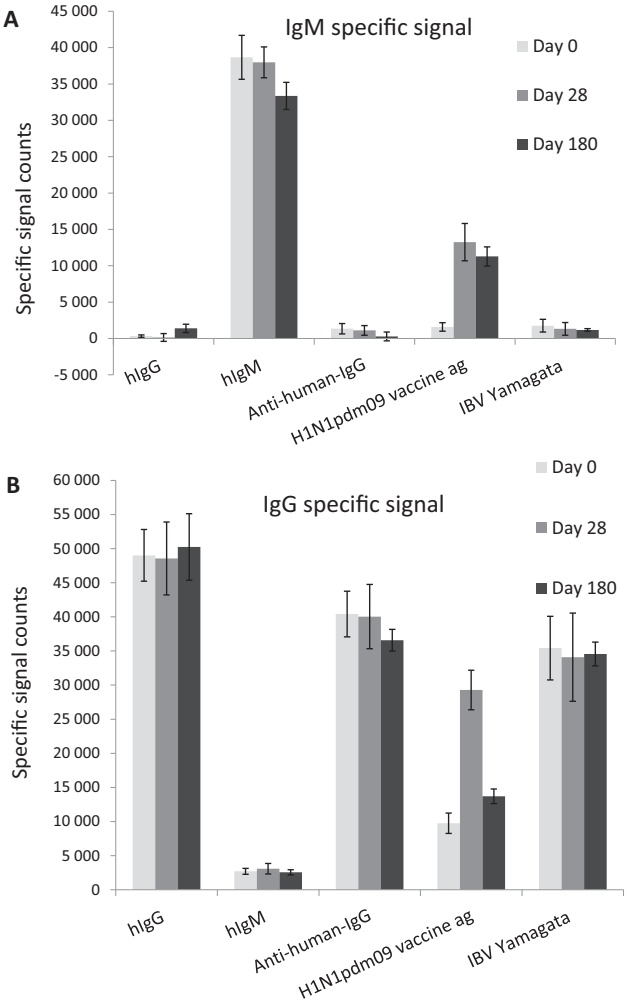

Fig. 2. Specific IgM and IgG MAIA signal counts from each spot in the microarray from a sample at day 0, day 28 and day 180 post-vaccination calculated as the mean signal from 2 duplicate spots in 2 duplicate wells (4 spots altogether) after subtracting the HSA signal.

specimens increased 2.9 to 4.2-fold in EIA and MAIA, respectively ($p < 0.001$). Late post-vaccination (day 180) IgG antibody levels declined 68% to 49% in EIA and MAIA, respectively ($p < 0.001$). The mean anti-IBV Yamagata IgM unit values stayed at a basal negative level of 15 to 16 units in pre- and post-vaccination samples (Fig. 3B). Mean anti-IBV (Yamagata strain) pre-existing IgG antibodies were at the level of 123 microarray units and showed no significant change after Pandemrix (IAV H1N1pdm09) vaccination. This confirms that the rise of antibodies against H1N1pdm09 vaccine ag is solely vaccine antigen-specific.

Fig. 4 shows the distribution of IgM and IgG unit values against H1N1pdm09 vaccine ag as measured by EIA and MAIA tests. Calculated cut-off values discriminate seronegative from seropositive serum samples. There were a few individuals who had pre-existing IgM antibodies against H1N1pdm09 vaccine ag before the vaccination. Most of the vaccinated individuals showed a significant rise in anti-H1N1pdm09 vaccine ag IgM and IgG antibodies.

3.3. Correlation between EIA, MAIA and HI titers

Next, we compared how EIA and MAIA antibody unit values correlated with each other. EIA and MAIA results had a very high degree of correlation for both IgM and IgG unit values (Fig. 5,

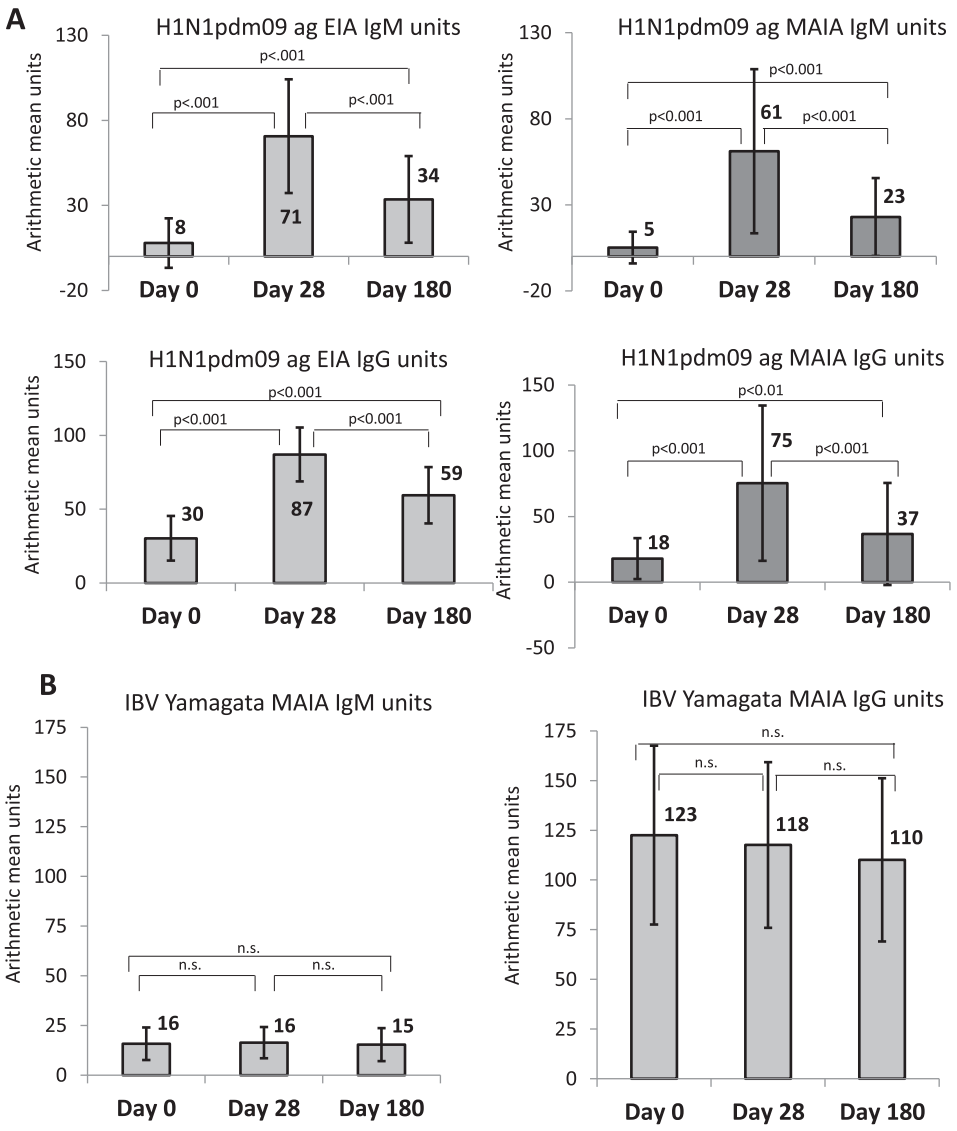

Fig. 3. The arithmetic mean IgM and IgG unit values against (A) H1N1pdm09 vaccine ag measured by EIA and MAIA and (B) IBV Yamagata measured by MAIA, in 60 individuals at day 0, day 28 and day 180 post-vaccination. The error bars indicate the standard deviations of the means (n = 4). The values are shown as microarray and enzyme immunoassay units, calculated in relation to the values for negative control specimens (MAIA or EIA unit values set as 0) and highly positive serum specimen (MAIA or EIA unit values set as 100).

$p < 0.001$). However, there was a tendency for a higher correlation for IgM unit values (n = 180, r = 0.910, $R^2 = 0.8271$, $p < 0.001$) than that of IgG unit values (n = 180, r = 0.546, $R^2 = 0.2981$, $p < 0.001$).

Then, we calculated the correlation between endpoint titers determined by three different methods (Fig. 6). We found that there was an almost perfect agreement between IgM endpoint titers determined by EIA and MAIA (n = 180, r = 0.943, $R^2 = 0.8891$, $p < 0.001$) and a very good agreement between IgM endpoint titers determined by either EIA (n = 180, r = 0.581, $R^2 = 0.3369$, $p < 0.001$) or MAIA and HI antibody titers (n = 180, r = 0.537, $R^2 = 0.285$, $p < 0.001$). The HI titer of 40, which is considered as a level of protective immunity, was considered to be equivalent to the IgM EIA titer of 180 and IgM MAIA titer of 150 (Fig. 6A).

There was also a strong, positive correlation between EIA and MAIA IgG endpoint titers, which was statistically significant (n = 180, r = 0.797, $R^2 = 0.6375$, $p < 0.001$). The antibody titers determined by HI also correlated highly significantly with IgG endpoint titers determined by EIA (n = 180, r = 0.779, $R^2 = 0.6063$, $p < 0.001$) and by MAIA (n = 180, r = 0.478, $R^2 = 0.2304$, $p < 0.001$). The HI titer of 40 was considered to be equivalent to the IgG EIA titer of 13,360 and IgG MAIA titer of 2980 (Fig. 6B).

3.4. Geometric mean titers and seroprotection rates

Geometric mean titers (GMTs) obtained with either EIA or MAIA were typically higher than those seen in the HI test (Table 1). Pre-vaccination anti-H1N1pdm09 vaccine ag antibody titers were relatively low. Four weeks after the vaccination, GMTs for all three assays increased significantly ($p < 0.001$). As analyzed by the HI test H1N1pdm09 vaccine-induced antibody responses between pre-vaccine and 28-day post-vaccine serum specimens increased 30.6-fold. Vaccine-induced IgM and IgG antibody GMTs rose 8.4 and 5.3-fold for MAIA, and 11.3 and 7.1-fold for EIA, respectively. It is noteworthy that there was also a significant decrease in the antibody GMT levels between day 28 and day 180 as analyzed by all different methods ($p < 0.0001$).

Next, we analyzed the rate of seroprotection i.e. the percentage of individuals showing $\geq$ 1:40 titer in the HI test and

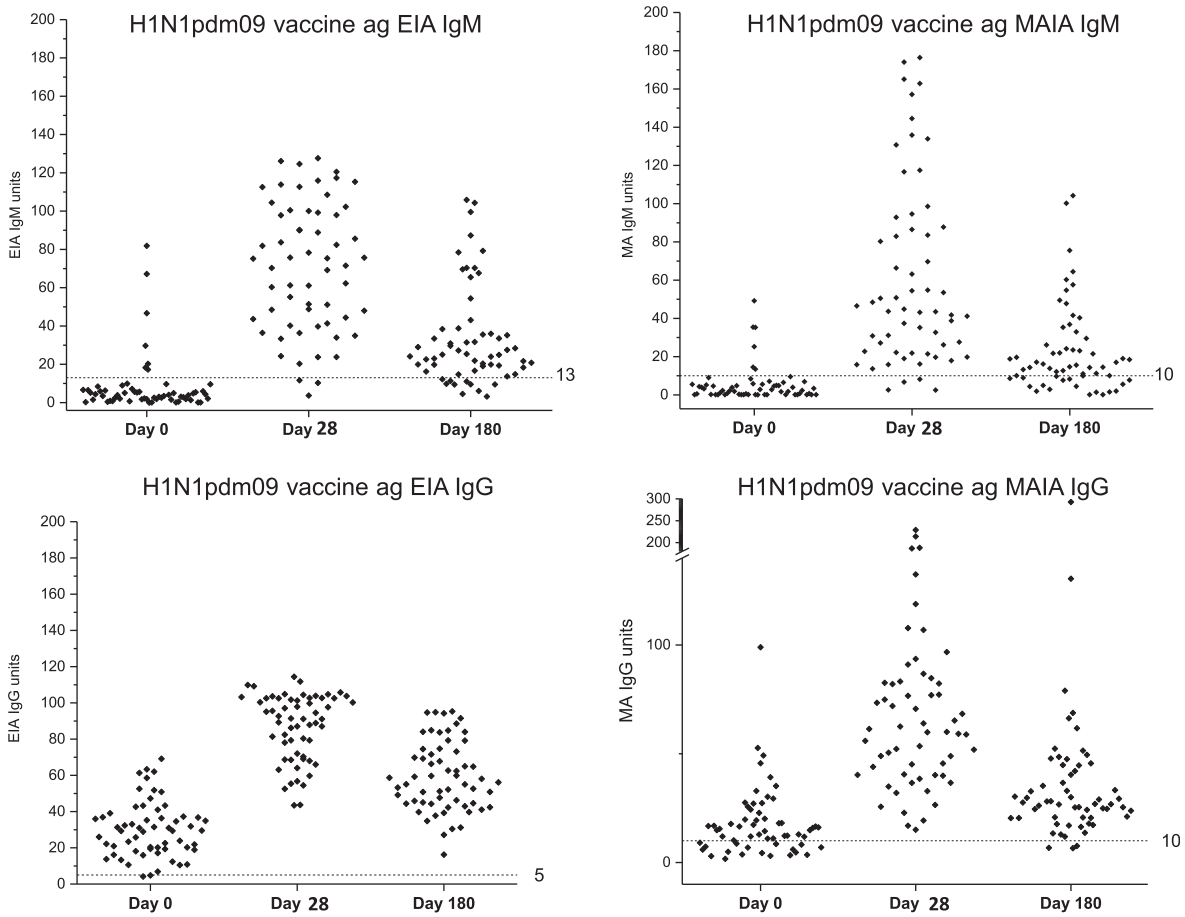

Fig. 4. Detection of IgM and IgG unit values against H1N1pdm09 vaccine ag in 60 individuals at day 0, day 28 and day 180 post-vaccination by EIA and MAIA. The dotted horizontal lines indicate the cut-off values.

corresponding titers in the EIA and MAIA, before and after the influenza vaccination (Table 2). Pandemrix vaccination induced very high seroprotection rates which ranged from 90 to 98% as measured by the HI, EIA, and MAIA tests. In EIA and MAIA IgG pre-vaccination seroprotective values were seen in 18–20% of individuals, respectively, whereas IgM pre-vaccination seroprotective values were detected in 7% of individuals by both methods. The seroprotection rate before the vaccination was 13% in the HI test. H1N1pdm09 virus-specific antibody levels remained at a theoretically seroprotective level in 80% of individuals by HI test and in 73–83% by EIA and MAIA at six months after the vaccination.

4. Discussion

Vaccines are typically developed and evaluated based on their ability to induce vaccine antigen-specific antibodies. It is generally assumed that antigen-specific antibody levels correlate with the protection of the host if the vaccine antigen(s) is/are the target(s) for neutralizing antibodies. In the present study, we used the traditional HI test together with EIA and MAIA tests to analyze antibody levels before and after vaccination with Pandemrix (H1N1pdm09) vaccine. The aim of the study was to evaluate Pandemrix vaccine-induced anti-H1N1pdm09 vaccine ag IgG and IgM antibody responses, antibody decline and the correlation of different influenza A virus-specific antibody assays. Especially, we also wanted to evaluate the potential of a novel multiplex microarray immunoassay for application in vaccine immunology research.

Currently, the HI test is widely used in influenza epidemiological and vaccine studies. The clinical protection threshold has been established for the assay [12,13,39] and the current policy requires that influenza vaccines induce serum HI titers of $\geq$40. It is generally considered that an individual having HI antibody titer of $\geq$40 is protected from influenza infection or at least symptomatic clinical illness. The HI test is relatively cheap and simple to perform and the assay does not require expensive equipment. Even though the HI test correlates well with the protection, there are several challenges with the assay. The results of the assay are read visually by the researcher and the determination of the end-point titer may be subjective. International standardization studies have shown a large variation in HI titers between different laboratories [40,41]. Also, the limiting features of the HI assay are its inability to distinguish between different antibody classes and to detect antibody levels with a high degree of specificity due to its titration-based test principle. The availability of different animal red blood cells may be limited and certain influenza A virus strains fail to agglutinate the most commonly used red blood cells.

EIA is a widely used assay to detect antigen-specific antibodies and it is a very well-validated method. However, the assay is limited by its ability to measure only a single antibody isotype against a single antigen in each assay. Even if EIA is of a relatively low cost and simple to perform, the required time to analyze a large number of samples practically limits the performance of EIA assays.

Multiplex immunoassay allows the analysis of antibody responses simultaneously against several different antigens in a large number of samples relative quickly. Therefore, multiplex immunoassay is a very good alternative to conventional EIA methods. Multiplex immunoassay method has the capability to dramatically simplify population-based large seroprevalence studies and

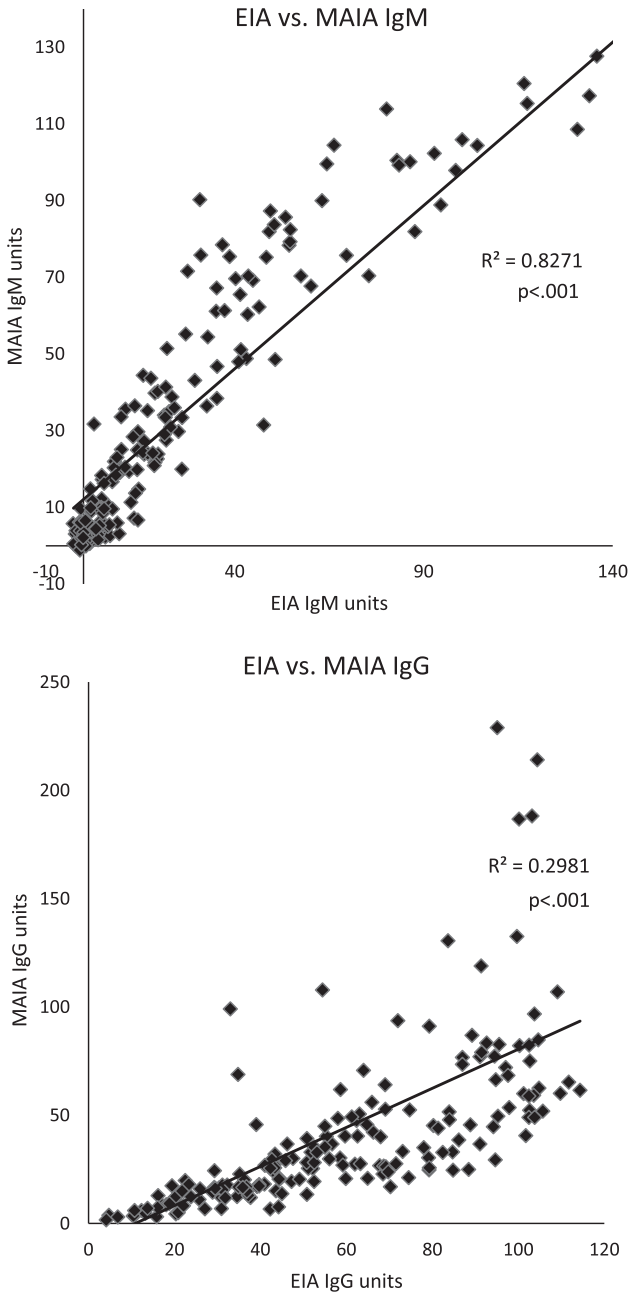

Fig. 5. Correlation of the multiplexed MAIA with the reference EIA assays for detection anti- H1N1pdm09 vaccine ag antibodies (N = 180). (A) Correlation between anti- H1N1pdm09 vaccine ag IgM unit values by EIA and MAIA; (B) Correlation between anti- H1N1pdm09 vaccine ag IgG unit values by EIA and MAIA. The results of both correlations were statistically significant and were greater or equal to r(180) = 0.546, R2 = 0.2981, p < 0.001, two-tailed.

to provide critical data to researchers working on vaccine development. Different types of multiplex immunoassays developed for simultaneous detection of vaccine-induced IgG antibodies have already proved their efficacy [42–44].

Previous analyses of vaccine-induced antibody responses have mainly focused on analyzing vaccine-specific IgG responses, which represents long-lasting immunity. Many assays such as the HI and neutralization tests do not discriminate antibody responses in different immunoglobulin classes. Antigen-specific IgM responses may play an important role in vaccine-induced immunity. Analysis of IgM responses as demonstrated in our EIA or MAIA tests provides a valuable addition to a traditional determination of post-vaccination immune responses. Our data revealed that most Pandemrix vaccinated individuals showed a strong anti-H1N1pdm09 vaccine ag IgM response that persisted even up to 6 months after the vaccination. It may be that virus-specific IgM antibodies, which cannot be separately measured by the HI or NT tests may contribute to protection against IAV more than previously thought.

We analyzed antibody levels in 60 influenza A virus vaccinated individuals by three different methods. In general, anti-influenza A antibody end-point titers detected by the HI test, EIA and MAIA correlated very well with each other. There was a better correlation between EIA and MAIA, compared to HI and EIA, and HI and MAIA which is likely due to the fact that the HI test is unable to differentiate between the antibody classes and it likely measures preferentially virus surface glycoprotein-specific antibodies while EIA and MAIA detect antibodies against all structural proteins of the H1N1pdm09 virus. Other studies have also reported a good correlation between EIA and multiplex assays [45,46]. We observed somewhat higher antibody unit values detected by EIA compared to those seen in MAIA. However, both methods showed a strong correlation for the arithmetic mean antibody units before and after the vaccination. It is important to consider potential interactions between different antibody classes and antigens in the same sample in microarray immunoassay. Our study showed that there was practically no cross-reactivity between IgM and IgG antibodies and different virus antigens despite simultaneous detection in one assay well. IBV Yamagata was included as one of the control antigens in MAIA to demonstrate the multiplex analytic capacity of the assay and to confirm that induced antibody levels are highly vaccine ag-specific. Anti-IBV Yamagata IgM and IgG antibodies remained at similar levels before and after Pandemrix vaccination. Thus, MAIA has a strong advantage over EIA since it includes several internal controls in the assay to ensure better assay quality and reliability of the assay results.

We also analyzed the performance of EIA and MAIA in estimating the theoretical "seroprotection" rates induced by the Pandemrix vaccine. We determined the EIA and MAIA titers that corresponded to the HI titer of 40 and estimated seroprotection rates by different assays. All methods showed very similar seroprotection rates before and after the vaccination. It is noteworthy that only 7% of individuals showed "seroprotective" IgM and ca. 20% of IgG antibody levels before vaccination. Three weeks after the vaccination IgM and IgG "seroprotection" values were seen in ca. 90% and 93–97% of cases, respectively. These figures were very similar to those seen by the HI test where 13% showed seroprotective antibody levels before the vaccination and 92% 4 weeks after the vaccination. Our results indicate that the Pandemrix vaccine induced strong antibody responses with very high theoretical seroprotection rates that were similarly detected with different analytical methods. However, it is worth mentioning that actual seroprotection was not measured. The EIA and MAIA seroprotective titers were determined in comparison to HI titers and these assays require their own validation to determine the actual seroprotection threshold.

To date, microarray technology is used in many research laboratories, but the assay(s) may one day emerge as routine clinical laboratory tests. The multiplex microarray immunoassay overcomes the limiting features of traditional immunoassays allowing the detection of multiple analytes and antibody types simultaneously in a small sample volume. This may be very critical in analyzing samples from infants. In addition to saving the sample volume and reagents, multiplex technology provides a significant save of time to complete the assay. The limitation of the multiplex technology is that it requires investment in expensive equipment and disposable supplies. Nevertheless, if four or more analytes are measured simultaneously the overall multiplex assay costs are lower than those of separate EIAs [47].

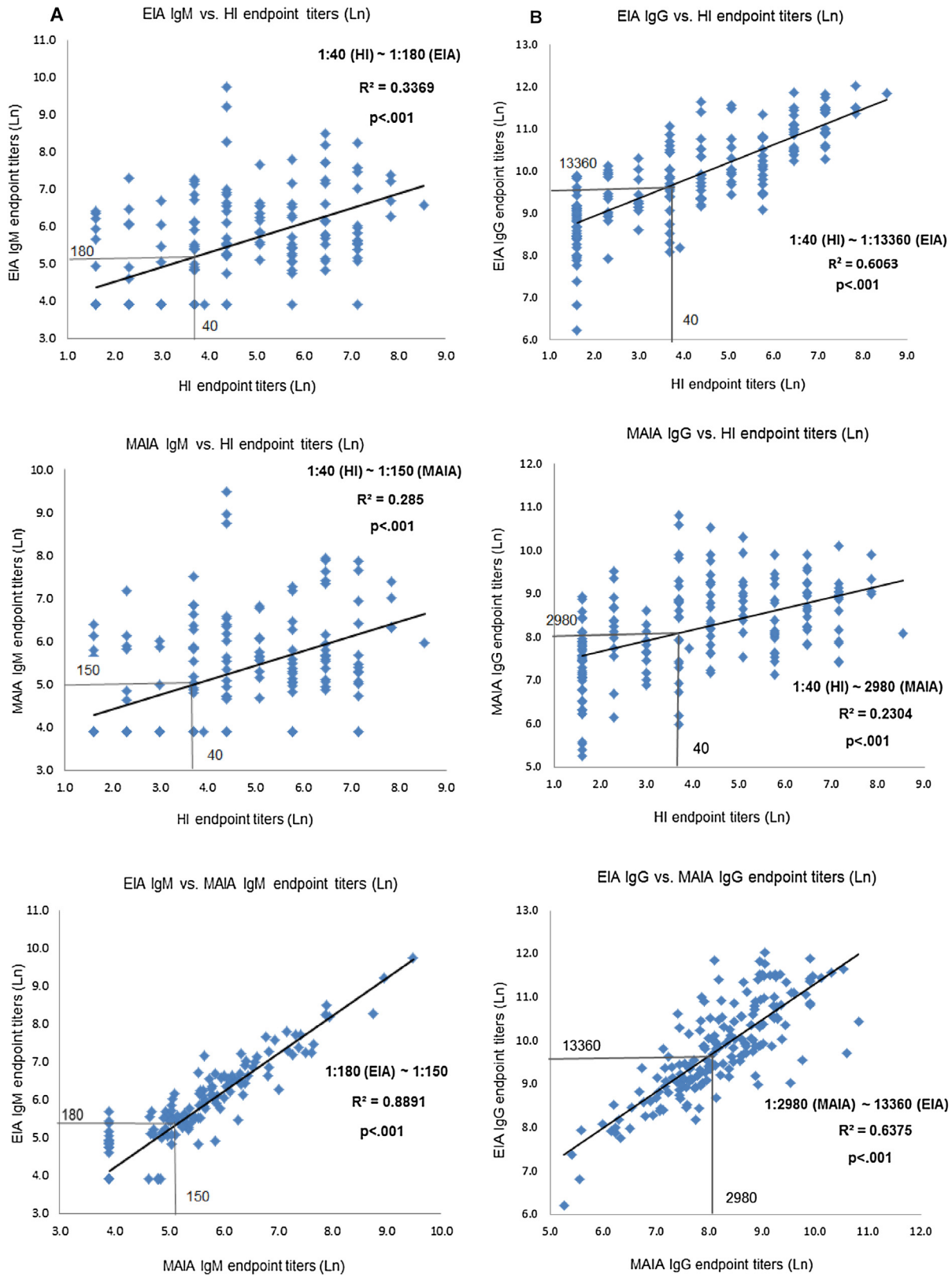

Fig. 6. Correlation of the antibody endpoint titers to A/California/07/2009 vaccine virus or H1N1pdm09 vaccine ag measured by haemagglutination inhibition (HI) test, enzyme immunoassay (EIA) and microarray immunoassay (MAIA). Natural logarithm (Ln)-transformed antibody titers are presented, trend lines and coefficient of determinations were calculated by linear regression analysis. The total number of serum samples is 180. (A) Correlations between HI and IgM EIA, HI and IgM MAIA, IgM EIA and IgM MAIA endpoint titers. (B) Correlations between HI and IgG EIA, HI and IgG MAIA, IgG EIA and IgG MAIA endpoint titers.

In the present study, we demonstrated that vaccination against the 2009 pandemic influenza A virus induces strong virus-specific IgM and IgG antibody responses likely providing a very high seroprotection rate against IAV H1N1pdm09. We also show that the microarray immunoassay data correlates extremely well with other conventional EIA and HI tests in estimating influenza A

Table 1
Pandemrix vaccine-induced anti-A/California/07/2009 virus antibody end-point titers as measured by haemagglutination inhibition (HI) test and anti-H1N1pdm09 vaccine ag (split whole virus) antibody titers as measured by enzyme immunoassay (EIA) and microarray immunoassay (MAIA).

Assay method	HI	EIA IgM	MAIA IgM	EIA IgG	MAIA IgG
Geometric mean titer [95% CI]					
Day 0	8.2	61.4	59.7	6700	1590
	[6.7–10.1]	[52.6–71.7]	[52.1–68.5]	[5494–8177]	[1255–2019]
Day 28	251	693	503	47,390	8350
	[167.3–376.7]	[518.0–928.5]	[375.3–676.2]	[39540–56810]	[7014–9943]
Fold increase*	30.6	11.3	8.4	7.1	5.3
Day 180	99.6	213	158	19,260	3510
	[67.0–148.1]	[171.3–266.9]	[125.1–200.2]	[16240–22850]	[2900–4240]
Fold increase*	12.1	3.5	2.6	2.9	2.2

The significance of differences between day 0 and 28 (increase) and day 28 and 180 (decrease) geometric mean titers within all different methods $p < 0.0001$.
* Fold increase compared to day 0.

Table 2
Theoretical seroprotection rates before and after vaccination with Pandemrix vaccine measured by haemagglutination inhibition (HI) test, enzyme immunoassay (EIA) and microarray immunoassay (MAIA).

	Number (%) of seroprotected* individuals (n = 60)						
	HI	EIA			MAIA		
		IgM	IgG	IgM + IgG	IgM	IgG	IgM + IgG
Day 0	8 (13%)	4 (7%)	11 (18%)	13 (22%)	4 (7%)	12 (20%)	14 (23%)
Day 28	55 (92%)	55 (92%)	58 (97%)	59 (98%)	54 (90%)	56 (93%)	59 (98%)
Day 180	48 (80%)	37 (62%)	37 (62%)	50 (83%)	33 (55%)	34 (57%)	44 (73%)

* Seroprotection relates to serum specimens with a HI titer ≥ 40, which corresponds to ≥180 and ≥13,360 in IgM and IgG EIA, respectively and ≥150 and ≥2980 in IgM and IgG MAIA assay, respectively.

virus-specific antibodies. We have thus shown that the microarray immunoassay is a very promising new tool to measure influenza vaccine as well as basically any other vaccine-induced antibody responses and it is also well suitable for large-scale seroepidemiological studies.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgements

We are grateful to Riitta Santanen for performing the HI assay. We also thank the THL clinical staff who collected the samples and all the volunteers who took part in this study.

Author's contributions

All authors meet the ICMJE criteria for authorship. AK developed MAIA, performed EIA and MAIA, analyzed serological data and wrote the manuscript together with IJ. TZ, RS and IJ were involved in the Pandemrix vaccination trial and sample collection. AK, LK, HP, TS, MW and IJ were involved in the study design and MAIA development and writing of the manuscript. All authors have read and approved the final version of the manuscript.

Ethics statement

The serum specimens analyzed in the present study were a subcohort of a larger study (Syrjänen et al., 2014) conducted by the National Institute for Health and Welfare (THL) in the city of Tampere, Finland. All participants gave their written informed consent for the study. The study protocols, sample collection, and consents were approved by the Ethics Committee of Pirkanmaa Health District, Finland (Permission ETL R0952M) and the study had received a THL protocol code AH1N1-483-09THL and an European Union clinical trials database code of EudraCT 2009-015700-26 and ClinicalTrials.gov codes NCT01024725.

Funding source

The study was supported by THL, University of Turku, Ministry of Social Affairs and Health, Finland and the Medical Research Council of the Academy of Finland. The funding organizations have no role in the design of the study and data analysis and presentation.

References

[1] Fineberg HV. Pandemic preparedness and response — lessons from the H1N1 Influenza of 2009. N Engl J Med 2014;370:1335–42. https://doi.org/10.1056/NEJMra1208802.
[2] Simona Puzelli, Livia Di Trani, Concetta Fabiani LC, Maria Alessandra De Marco, Ilaria Capua JFA, Maria Zambon and ID. Serological analysis of serum samples from humans exposed to avian H7 influenza viruses in Italy between 1999 and 2003. J Infect Dis; 2005. doi:192:1318–22.
[3] Shu LL, Zhou NN, Sharp GB, He SQ, Zhang TJ, Zou WW, et al. An epidemiological study of influenza viruses among Chinese farm families with household ducks and pigs. Epidemiol Infect 1996;117:179–88. https://doi.org/10.1017/s0950268800001291.
[4] Yamazaki Y, Doy M, Okabe N, Yasui Y, Nakashima K, Fujieda T, et al. Serological survey of avian H5N2-subtype influenza virus infections in human populations. Arch Virol 2009;154:421–7. https://doi.org/10.1007/s00705-009-0319-7.
[5] Sikkema RS, Freidl GS, de Bruin E, Koopmans M. Weighing serological evidence of human exposure to animal influenza viruses - a literature review. Euro Surveill 2016;21. https://doi.org/10.2807/1560-7917.ES.2016.21.44.30388.
[6] Webster RG. The importance of animal influenza for human disease. Vaccine 2002;20(Suppl 2):S16–20.
[7] Trombetta C, Perini D, Mather S, Temperton N, Montomoli E, Trombetta CM, et al. Overview of serological techniques for influenza vaccine evaluation: past, present and future. Vaccines 2014;2:707–34. https://doi.org/10.3390/vaccines2040707.
[8] Plotkin SA, Orenstein WA, Offit PA. Vaccines. n.d.
[9] Pedersen JC. Hemagglutination-inhibition assay for influenza virus subtype identification and the detection and quantitation of serum antibodies to influenza virus. Methods Mol Biol 2014;1161:11–25. https://doi.org/10.1007/978-1-4939-0758-8_2.

[10] Haveri A, Ikonen N, Kantele A, Anttila V-J, Ruotsalainen E, Savolainen-Kopra C, et al. Seasonal influenza vaccines induced high levels of neutralizing cross-reactive antibody responses against different genetic group influenza A(H1N1) pdm09 viruses. Vaccine 2019;37:2731–40. https://doi.org/10.1016/j.vaccine.2019.03.078.

[11] Baldo V, Baldovin T, Floreani A, Minuzzo M, Trivello R. Response to influenza vaccine in people with non-protective HI antibody titers. Eur J Epidemiol 2006;21:843–5. https://doi.org/10.1007/s10654-006-9071-4.

[12] de Jong JC, Palache AM, Beyer WEP, Rimmelzwaan GF, Boon ACM, Osterhaus ADME. Haemagglutination-inhibiting antibody to influenza virus. Dev Biol (Basel) 2003;115:63–73.

[13] Coudeville L, Bailleux F, Riche B, Megas F, Andre P, Ecochard R. Relationship between haemagglutination-inhibiting antibody titres and clinical protection against influenza: development and application of a bayesian random-effects model. BMC Med Res Methodol 2010;10:18. https://doi.org/10.1186/1471-2288-10-18.

[14] Tighe PJ, Ryder RR, Todd I, Fairclough LC. ELISA in the multiplex era: potentials and pitfalls. Proteomics Clin Appl 2015;9:406–22. https://doi.org/10.1002/prca.201400130.

[15] Ayling K, Vedhara K, Fairclough L. Measuring vaccine responses in the multiplex era. Methods Mol Biol 2018;1781:327–40. https://doi.org/10.1007/978-1-4939-7828-1_17.

[16] Jorquera PA, Mishin VP, Chesnokov A, Nguyen HT, Mann B, Garten R, et al. Insights into the antigenic advancement of influenza A(H3N2) viruses, 2011–2018. Sci Rep 2019;9:2676. https://doi.org/10.1038/s41598-019-39276-1.

[17] Schena M, Shalon D, Davis RW, Brown PO. Quantitative monitoring of gene expression patterns with a complementary DNA Microarray. Science (80-) 1995;270:467–70. https://doi.org/10.1126/science.270.5235.467.

[18] Yasrebi H. Comparative study of joint analysis of microarray gene expression data in survival prediction and risk assessment of breast cancer patients. Brief Bioinform 2016;17:771–85. https://doi.org/10.1093/bib/bbv092.

[19] Selvarajah S, Negm OH, Hamed MR, Tubby C, Todd I, Tighe PJ, et al. Development and validation of protein microarray technology for simultaneous inflammatory mediator detection in human sera. Mediators Inflamm 2014;2014:1–12. https://doi.org/10.1155/2014/820304.

[20] Negm OH, Hamed MR, Dilnot EM, Shone CC, Marszalowska I, Lynch M, et al. Profiling humoral immune responses to clostridium difficile-specific antigens by protein microarray analysis. Clin Vacc Immunol 2015;22:1033–9. https://doi.org/10.1128/CVI.00190-15.

[21] Yuk C-S, Lee HK, Kim HT, Choi YK, Lee BC, Chun B-H, et al. Development and evaluation of a protein microarray chip for diagnosis of hepatitis C virus. Biotechnol Lett 2004;26:1563–8. https://doi.org/10.1023/B:BILE.0000045653.92719.a4.

[22] Schepp RM, De Haan CAM, Wilkins D, Layman H, Graham BS, Esser MT, et al. Development and standardization of a high-throughput multiplex immunoassay for the simultaneous quantification of specific antibodies to five respiratory syncytial virus proteins. MSphere 2019;4. https://doi.org/10.1128/mSphere. 00236-19.

[23] Itell HL, McGuire EP, Muresan P, Cunningham CK, McFarland EJ, Borkowsky W, et al. Development and application of a multiplex assay for the simultaneous measurement of antibody responses elicited by common childhood vaccines. Vaccine 2018;36:5600–8. https://doi.org/10.1016/J.VACCINE.2018.07.048.

[24] Engin ED. The use of multiplexing technology in the immunodiagnosis of infectious agents. J Immunoass Immunochem 2019;40:109–22. https://doi.org/10.1080/15321819.2018.1563551.

[25] Price JV, Jarrell JA, Furman D, Kattah NH, Newell E, Dekker CL, et al. Characterization of influenza vaccine immunogenicity using influenza antigen microarrays. PLoS ONE 2013;8. https://doi.org/10.1371/JOURNAL.PONE.0064555.

[26] Gall A, Hoffmann B, Harder T, Grund C, Hoper D, Beer M. Design and validation of a microarray for detection, hemagglutinin subtyping, and pathotyping of avian influenza viruses. J Clin Microbiol 2009;47:327–34. https://doi.org/10.1128/JCM.01330-08.

[27] Bucukovski J, Latorre-Margalef N, Stallknecht DE, Miller BL. A multiplex label-free approach to avian influenza surveillance and serology. PLoS ONE 2015;10:e0134484. https://doi.org/10.1371/journal.pone.0134484.

[28] Nakajima R, Supnet M, Jasinskas A, Jain A, Taghavian O, Obiero J, et al. Protein microarray analysis of the specificity and cross-reactivity of influenza virus hemagglutinin-specific antibodies. MSphere 2018;3. https://doi.org/10.1128/msphere.00592-18.

[29] Meade P, Latorre-Margalef N, Stallknecht DE, Krammer F. Development of an influenza virus protein microarray to measure the humoral response to influenza virus infection in mallards. Emerg Microbes Infect 2017;6:e110. https://doi.org/10.1038/emi.2017.98.

[30] Freidl GS, de Bruin E, van Beek J, Reimerink J, de Wit S, Koch G, et al. Getting more out of less – a quantitative serological screening tool for simultaneous detection of multiple influenza A hemagglutinin-types in chickens. PLoS ONE 2014;9:e108043. https://doi.org/10.1371/journal.pone.0108043.

[31] Desbien AL, Van Hoeven N, Reed SJ, Casey AC, Laurance JD, Baldwin SL, et al. Development of a high density hemagglutinin protein microarray to determine the breadth of influenza antibody responses. Biotechniques 2013;54. https://doi.org/10.2144/000114041.

[32] Stevens J, Blixt O, Paulson JC, Wilson IA. Glycan microarray technologies: tools to survey host specificity of influenza viruses. Nat Rev Microbiol 2006;4:857–64. https://doi.org/10.1038/nrmicro1530.

[33] Walker WT, Faust SN. Monovalent inactivated split-virion AS03-adjuvanted pandemic influenza A (H1N1) vaccine. Expert Rev Vaccines 2010;9:1385–98. https://doi.org/10.1586/erv.10.141.

[34] Syrjänen RK, Jokinen J, Ziegler T, Sundman J, Lahdenkari M, Julkunen I, et al. Effectiveness of pandemic and seasonal influenza vaccines in preventing laboratory-confirmed influenza in adults: a clinical cohort study during epidemic seasons 2009–2010 and 2010–2011 in Finland. PLoS ONE 2014;9:e108538. https://doi.org/10.1371/journal.pone.0108538.

[35] Kale V, Päkkilä H, Vainio J, Ahomaa A, Sirkka N, Lyytikäinen A, et al. Spectrally and spatially multiplexed serological array-in-well assay utilizing two-color upconversion luminescence imaging. Anal Chem 2016;88:4470–7. https://doi.org/10.1021/acs.analchem.6b00337.

[36] Anna Kazakova, Laura Kakkola, Henna Päkkilä, Tamara Teros-Jaakkola, Tero Soukka, Ville Peltola, et al. Serological array-in-well multiplex assay reveals a high rate of respiratory virus infections and reinfections in young children. MSphere 2019.

[37] World Health Organization. WHO Global Influenza Surveillance Network. Manual for the Laboratory Diagnosis and Virological Surveillance of Influenza. World Health Organization; 2011.

[38] Kutsaya A, Teros-Jaakkola T, Kakkola L, Toivonen L, Peltola V, Waris M, et al. Prospective clinical and serological follow-up in early childhood reveals a high rate of subclinical RSV infection and a relatively high reinfection rate within the first 3 years of life. Epidemiol Infect 2016:1–12. https://doi.org/10.1017/S0950268815003143.

[39] Hobson D, Curry RL, Beare AS, Ward-Gardner A. The role of serum haemagglutination-inhibiting antibody in protection against challenge infection with influenza A2 and B viruses. J Hyg (Lond) 1972;70:767–77. https://doi.org/10.1017/s0022172400022610.

[40] Wagner R, Göpfert C, Hammann J, Neumann B, Wood J, Newman R, et al. Enhancing the reproducibility of serological methods used to evaluate immunogenicity of pandemic H1N1 influenza vaccines—an effective EU regulatory approach. Vaccine 2012;30:4113–22. https://doi.org/10.1016/j.vaccine.2012.02.077.

[41] Wood JM, Major D, Heath A, Newman RW, Höschler K, Stephenson I, et al. Reproducibility of serology assays for pandemic influenza H1N1: collaborative study to evaluate a candidate WHO International Standard. Vaccine 2012;30:210–7. https://doi.org/10.1016/j.vaccine.2011.11.019.

[42] Smits GP, van Gageldonk PG, Schouls LM, van der Klis FRM, Berbers GAM. Development of a bead-based multiplex immunoassay for simultaneous quantitative detection of IgG serum antibodies against measles, mumps, rubella, and varicella-zoster virus. Clin Vaccine Immunol 2012;19:396–400. https://doi.org/10.1128/CVI.05537-11.

[43] Schepp RM, Berbers GAM, Ferreira JA, Reimerink JH, van der Klis FR. A novel multiplex poliovirus binding inhibition assay applicable for large serosurveillance and vaccine studies, without the use of live poliovirus. J Virol Methods 2017;241:15–23. https://doi.org/10.1016/J.JVIROMET.2016.12.006.

[44] Gageldonk PGM, van Schaijk FG, van der Klis FR, Berbers GAM. Development and validation of a multiplex immunoassay for the simultaneous determination of serum antibodies to Bordetella pertussis, diphtheria and tetanus. J Immunol Methods 2008;335:79–89. https://doi.org/10.1016/J.JIM.2008.02.018.

[45] Elshal M, Mccoy J. Multiplex bead array assays: Performance evaluation and comparison of sensitivity to ELISA. Methods 2006;38:317–23. https://doi.org/10.1016/j.ymeth.2005.11.010.

[46] Khan SS, Smith MS, Reda D, Suffredini AF, McCoy JP. Multiplex bead array assays for detection of soluble cytokines: comparisons of sensitivity and quantitative values among kits from multiple manufacturers. Cytometry 2004;61B:35–9. https://doi.org/10.1002/cyto.b.20021.

[47] Leng SX, McElhaney JE, Walston JD, Xie D, Fedarko NS, Kuchel GA. ELISA and multiplex technologies for cytokine measurement in inflammation and aging research. J Gerontol Ser A Biol Sci Med Sci 2008;63:879–84. https://doi.org/10.1093/gerona/63.8.879.