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Long-lasting heterologous antibody responses after sequential vaccination with A/Indonesia/5/2005 and A/Vietnam/1203/2004 pre-pandemic influenza A(H5N1) virus vaccines

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ABSTRACT

Background: Avian influenza A(H5N1) viruses have caused sporadic infections in humans and thus they pose a significant global health threat. Among symptomatic patients the case fatality rate has been ca. 50%. H5N1 viruses exist in multiple clades and subclades and several candidate vaccines have been developed to prevent A(H5N1) infection as a principal measure for preventing the disease.
Methods: Serum antibodies against various influenza A(H5N1) clade viruses were measured in adults by ELISA-based microneutralization and haemagglutination inhibition tests before and after vaccination with two different A(H5N1) vaccines in 2009 and 2011.
Results: Two doses of AS03-adjuvanted A/Indonesia/5/2005 vaccine induced good homologous but poor heterologous neutralizing antibody responses against different clade viruses. However, non-adjuvanted A/Vietnam/1203/2004 booster vaccination in 2011 induced very strong and long-lasting homologous and heterologous antibody responses while homologous response remained weak in naïve subjects.
Conclusions: Sequential vaccination with two different A(H5N1) pre-pandemic vaccines induced long-lasting high level cross-clade immunity against influenza A(H5N1) strains, thus supporting a prime-boost vaccination strategy in pandemic preparedness plans.

1. Introduction

Highly pathogenic avian influenza (HPAI) A(H5) viruses of the A/goose/Guangdong/1/96 haemagglutinin (HA) lineage emerged in 1997 [1]. These viruses have become enzootic in some countries, infecting wild birds and causing outbreaks in poultry and sporadic human infections. Between 2003 and 2020 the World Health Organization (WHO) has reported a total of 861 laboratory confirmed cases of A(H5N1) human infections from 17 countries, including 455 deaths [2]. Although avian H5N1 viruses have not gained the capacity for sustained human-to-human transmission, they may well develop this ability through multiple genetic changes.

To date 10 genetically distinct HA-based clades (0–9) and several subclades have been identified among A(H5N1) viruses [3]. Viruses from clades 0, 1, 2 and 7 have caused sporadic infections in humans, most commonly by clade 2 viruses [4]. Subclade 2.3.2.1a virus was detected in the most recent A(H5N1) human case in 2019 [5]. The H5 HPAI viruses have recently undergone reassortment with neuraminidase (NA) and internal genes from other viruses to replace N1 gene segment by N2, N3, N5, N6, N8 or N9 [6–9]. Concomitant with these reassortment events humans have been infected with the subtype H5N6 [10–12].

WHO coordinates the development of influenza candidate vaccine viruses (CVVs) for the purpose of pandemic preparedness. There has been a great deal of research on the immunogenicity of A(H5N1) CVVs in humans [13–31]. However, limited amount of data is available on vaccine-induced neutralizing antibody responses towards a wider range of A(H5N1) viruses [32–35]. For the present study we collected sera from volunteers before and after H5N1 influenza vaccinations in 2009 and 2011. In the present study we analyzed vaccine-induced humoral immune responses by MN tests against ten vaccine viruses belonging to different genetic clades and subclades of influenza A(H5N1). By microneutralization test we measured the duration of protective response and cross-reactivity up to 24 months after the heterologous booster vaccination. In addition, we analyzed the differences in immune responses to primary and booster vaccination to a limited number of virus strains using HI tests.

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2. Materials and methods

2.1. Vaccines and schedule

The vaccines administered in this independent (nonsponsored), occupational safety based study were pre-pandemic influenza vaccine (H5N1), inactivated, AS03-adjuvanted A/Indonesia/5/2005-like (3.75 μg of HA/dose) split virion vaccine (GlaxoSmithKline, lot number AA3BA020AA) in 2009 and Vepacel®, inactivated, non-adjuvanted A/Vietnam/1203/2004-like (7.5 μg of HA/dose) whole virion H5N1 vaccine (Baxter, lot number VNV1K005A) in 2011. The employer provided each volunteer the H5N1 influenza vaccines. Vaccines were administered intramuscularly (deltoid muscle). Two vaccine doses were given to each person in three week intervals. Serum samples were collected prior to vaccination on day 0, and the post-vaccination serum specimens were collected at days 21, 42, 182 (six months), 365 (one year, for 2011 vaccination only) and 730 (two years) after the vaccination with pre-pandemic influenza vaccines (Fig. 1).

2.2. Participants

Clinically healthy subjects were recruited on a voluntary basis from the staff of the Virology Unit at the Finnish Institute for Health and Welfare, Finnish Food Authority and Department of Medicine at Helsinki University Hospital. 11 men and 53 women, median age 40 years (range: 26–62), were involved in the 2009 vaccination trial. Two years later, at the time of the Vepacel® vaccination in 2011, 13 men and 48 women, median age 39 years (range: 19–68), participated in the study. 40 of the participants were included in both vaccination studies. All participants gave their informed written consent before enrolment in the study.

2.3. Viruses

Ten pre-pandemic influenza A (H5N1) candidate vaccine viruses were selected for serological analyses: A/Vietnam/1203/2004 (SJRG-161052, clade 1), A/duck/Hunan/795/2002 (SJRG-166614, clade 2.1.1), A/Indonesia/5/2005 (CDC-RG2, clade 2.1.3.2), A/whooper swan/Mongolia/244/2005 (SJRG-163243, clade 2.2), A/Egypt/N03072/2010 (IDCDC-RG29, clade 2.2.1), A/Egypt/3300-Namru3/2008 (IDCDC-RG13, clade 2.2.1.1), A/Hubei/1/2010 (IDCDC-RG30, clade 2.3.2.1a), A/Anhui/1/2005 (IBCDC-RG6, clade 2.3.4), A/goose/Guiyang/337/2006 (SJRG-165396, clade 4) and A/chicken/Vietnam/NCVD-016/2008 (IDCDC-RG12, clade 7.1). All viruses were propagated in MDCK cells and stored in aliquots at −70 °C.

2.4. Phylogenetic analysis of the HA gene

The phylogenetic analysis of the HA gene for selected virus strains was performed as described [36]. Mega (Molecular Evolutionary Genetics Analysis) software version 7.0 [37] was used in the construction of a phylogenetic tree. The Neighbor-joining method [38] with the maximum composite likelihood model [39] was used to generate the phylogenetic tree. Bootstrapping was performed with 1000 replicates [40]. HA sequences included for the phylogenetic tree were obtained from GISAID EpiFlu™-Database. Accession numbers of the sequences are A/Vietnam/1203/2004 (EPI361524), A/duck/Hunan/795/2002 (EPI135862), A/Indonesia/5/2005 (EPI116487), A/whooper swan/Mongolia/244/2005 (EPI227591), A/Egypt/N03072/2010 (EPI255379), A/Egypt/3300-Namru3/2008 (EPI165072), A/Hubei/1/2010 (EPI337231), A/Anhui/1/2005 (EPI101917), A/goose/Guiyang/337/2006 (EPI107811) and A/chicken/Vietnam/NCVD-016/2008 (EPI180243).

2.5. Serologic assays

Serum antibody levels were determined by microneutralization (MN) tests with the virus strains described above for 26 subjects who received two different vaccines. An ELISA-based MN test was performed according to the WHO guidelines [41], modified as previously described [42] yet optimized for a horseradish peroxidase (HRP) labeled influenza A nucleoprotein antibody (catalog no 7304; Medix Biochemica, Espoo, Finland) and incubation times used in this study. The antibody was diluted 1:500 and incubated at +37 °C for 1 h and the substrate o-phenylenediamine dihydrochloride (Sigma-Aldrich, USA) was incubated for 1 h at room temperature. The neutralizing endpoint was determined as previously described [41]. For statistical analyses, serum specimens with MN titers <10 and >1280 were assigned a titer value of 5 and 2560, respectively.

Haemagglutination inhibition (HI) tests were conducted with some of the vaccine virus strains described above. HI tests were performed according to WHO guidelines [41] using turkey erythrocytes (0.5%/vol). For statistical analyses, serum specimens with HI titers < 10 were assigned a titer value of 5.

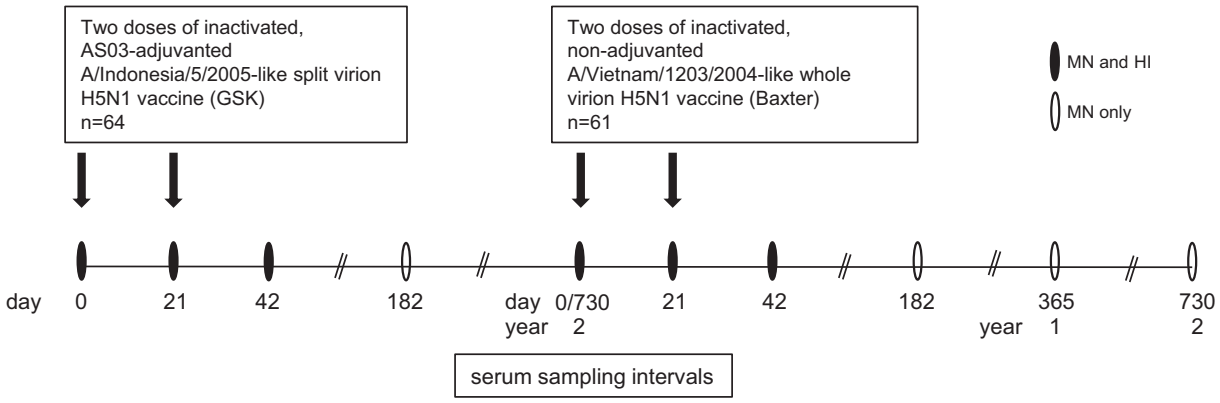

Fig. 1. A schematic presentation of study design. The study included 64 and 61 clinically healthy volunteers aged 26–62 and 19–68 years for AS03-adjuvanted A/Indonesia/5/2005-like split virion H5N1 vaccine (GSK) in 2009 and non-adjuvanted A/Vietnam/1203/2004-like whole virion H5N1 vaccine (Baxter) in 2011, respectively. 40 of the participants were included in both vaccination trials. The subjects received vaccinations at days 0 and 21 and follow-up samples were collected at days 21, 42, 182 (six months), 365 (one year, for 2011 only) and 730 (two years). The solid black dots indicate microneutralization (MN) and haemagglutination inhibition (HI) testing, white dots MN testing only.

2.6. Immunizations of rabbits and guinea pigs

In order to study the immunogenicity and the ability of the pre-pandemic influenza A(H5N1) vaccine to induce cross-reactive antibody responses in animals five guinea pigs and five rabbits were immunized for four times in three weeks intervals for A/Indonesia/5/2005 vaccine. Each animal received subcutaneously one human dose (3.75 μg HA) in AS03 adjuvant with each immunization. Serum samples from guinea pigs were collected before the first immunization (day 0) and one week after the last immunization (day 70). From the rabbits, serum samples were collected on day 0, before each immunization on days 21, 42, and 63, and one week after the last immunization on day 70. Immunizations of the animals and the collection of serum samples were approved by the Ethical Committee of Finnish Institute of for Health and Welfare (permission KTL 2008-02). Serum samples were stored at −20 °C and analyzed for influenza A virus specific antibodies by the HI test.

2.7. Statistical analysis

Geometric mean titers (GMT) with 95% confidence intervals and seroprotection rates (HI titer ≥ 1:40, MN titer ≥ 1:80) for each virus were calculated. Statistical differences between the groups were calculated using Student's *t*-test (paired, two-tailed) and the statistical significance level of difference was set to $p < 0.01$. HI and MN assays for A/Vietnam/1203/2004 vaccine virus were correlated and compared with Pearson and t-tests. Log-transformed titers were performed by linear regression analysis using Microsoft Excel 2010 software.

3. Results

Altogether 64 and 61 clinically healthy adults were enrolled into the AS03-adjuvanted A/Indonesia/5/2005-like split virion H5N1 vaccination (GSK) in 2009 and non-adjuvanted A/Vietnam/1203/2004-like whole virion H5N1 vaccination (Baxter) in 2011, respectively (Fig. 1). Of the volunteers two had a history of two doses of MF59®-adjuvanted A/Vietnam/1194/2004 H5N1 pre-pandemic influenza vaccine [19] 6–7 months before the first vaccination in 2009 and therefore, the data of these two vaccinees is shown separately. The pre-pandemic A(H5N1) vaccine viruses used in this study clustered in 4 genetic clades and several subclades (Fig. 2).

The antibody titers against A/Vietnam/1203/2004 vaccine virus were first determined by the HI test (Table 1). Immunologically naïve participants (seronegative) did not have pre-existing antibodies in 2009. Heterologous AS03-adjuvanted A/Indonesia/5/2005 vaccination induced GMTs from 5.1 to 5.5 and 6.4 three weeks after the first and the second vaccination, respectively. The two participants who had been vaccinated with MF59®-adjuvanted A/Vietnam/1194/2004 vaccine virus (clade 1) 6–7 months earlier had very strong booster responses compared to naïve individuals. The fold increases in GMT values were 32.0 and 1.1 three weeks after the first vaccination and 45.3 and 1.3 three weeks after the second vaccination for pre-vaccinated (n = 2) and naïve (n = 61) subjects, respectively.

In 2011 a non-adjuvanted A/Vietnam/1203/2004 H5N1 vaccine was used. Of the vaccinees, 21 subjects received their first H5N1 vaccination (Vietnam strain only) and 40 subjects had been vaccinated (receiving Indonesian strain) earlier in 2009. No pre-existing antibodies were detected for A/Vietnam/1203/2004 virus strain in any of the vaccinees. Heterologous H5N1 vaccination two years earlier with AS03-adjuvanted A/Indonesia/5/2005 vaccine gave rise to strong booster responses with non-adjuvanted A/Vietnam/1203/2004 H5N1 vaccine compared to naïve individuals (Table 1). GMTs of Indonesian strain pre-vaccinated subjects increased significantly three weeks after the first and the second vaccination, whereas homologous responses against the vaccine strain remained quite low among the naïve individuals. In the HI test the fold increase in GMT values were 36.1 and 1.7 three weeks after the first vaccination and 32.0 and 1.8 three weeks after the second vaccination for pre-vaccinated and naïve groups, respectively. A similar difference in favor for the pre-vaccinated group was seen when comparing the seroprotection rates (SR) of the groups in the HI test (Fig. 3).

Using vaccine virus A/Vietnam/1203/2004 as a reference strain, we found that the antibody titers of the HI and MN assays correlated strongly positively (n = 161, r = 0.9585, R^2 = 0.9409, $p < 0.0001$). An HI titer of 40 was considered to be equivalent to a MN titer of 80 (Fig. 4). This value was used below for all studied A(H5N1) viruses as a measure for seroprotection.

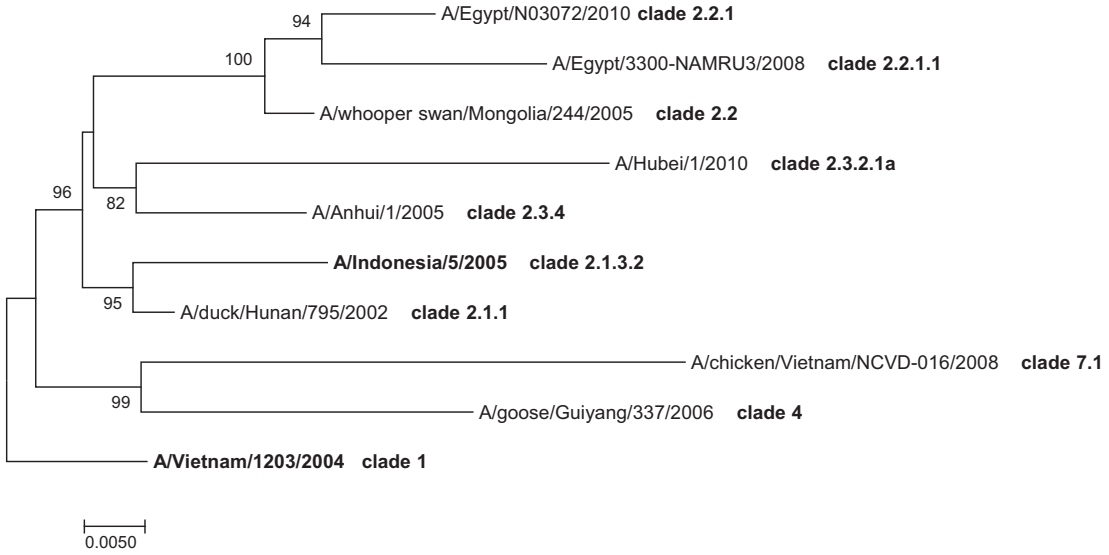

Fig. 2. Phylogenetic tree of the haemagglutinin (HA) sequences of influenza A(H5N1) candidate vaccine viruses used in this study. Sequences used in the phylogenetic analysis included 1643 nucleotides of the HA gene without the signal peptide. The tree was constructed by the Neighbor-joining method with Mega software version 7.0. The viruses used in vaccinations are highlighted in bold type. The horizontal lines are proportional to the number of nucleotide changes. Different clades are marked.

Table 1
Vaccine-induced humoral immune responses against influenza A(H5N1) vaccine strain A/Vietnam/1203/2004 measured by haemagglutination inhibition (HI) test. 64 and 61 subjects received two doses of AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine (GSK) and non-adjuvanted A/Vietnam/1203/2004-like H5N1 vaccine (GSK) in 2009 and 2011, respectively. Within the 2009 vaccinated group two subjects had received two doses of MF59®-adjuvanted A/Vietnam/1194/2004 H5N1 vaccine (Novartis) 6–7 months prior receiving the A/Indonesia/5/2005-like H5N1 vaccine (GSK). Of 2011 vaccinated group 21 subjects received their first A(H5N1) vaccinations (Vietnam strain only) and 39 subjects were vaccinated earlier in 2009 with A/Indonesia/5/2005-like H5N1 vaccine (GSK). Day 0 refers to serum samples collected before the vaccination. Statistical differences between the pre-vaccine (day 0) and post-vaccine (days 21 and 42) antibody levels were calculated using Student's *t*-test. CI: confidence interval. Statistical comparison to day 0 GMT values,*p < 0.01 **p < 0.001 ***p < 0.0001.

Vaccination	**2 × Indonesia/5/05**		**2 × Indonesia/5/05 and 2 × Vietnam/1203/04**		**2 × Vietnam/1203/04**		**2 × Vietnam/1194/04 and 2 × Indonesia/5/05**	
Geometric mean titer [95% CI] *Fold increase*								
day 0	5.1	[5.0–5.3] n = 60	5.1	[4.9–5.3] n = 39	5.1	[4.9–5.3] n = 21	10.0	[2.6–38.9] n = 2
day 21	5.5 *1.1*	[5.1–5.9] n = 61	183.8 *36.1*	[86.1–392.2] *** n = 30	8.9 *1.7*	[4.9–16.1] n = 18	320.0 *32.0*	[320.0–320.0] n = 2
day 42	6.4 *1.3*	[5.7–7.2] ** n = 58	163.1 *32.0*	[81.6–325.9] *** n = 36	9.4 *1.8*	[5.5–16.0] n = 21	452.5 *45.3*	[229.4–892.6] n = 2

Statistical comparison to day 0 GMT values, *p < 0.01 **p < 0.001 ***p < 0.0001.

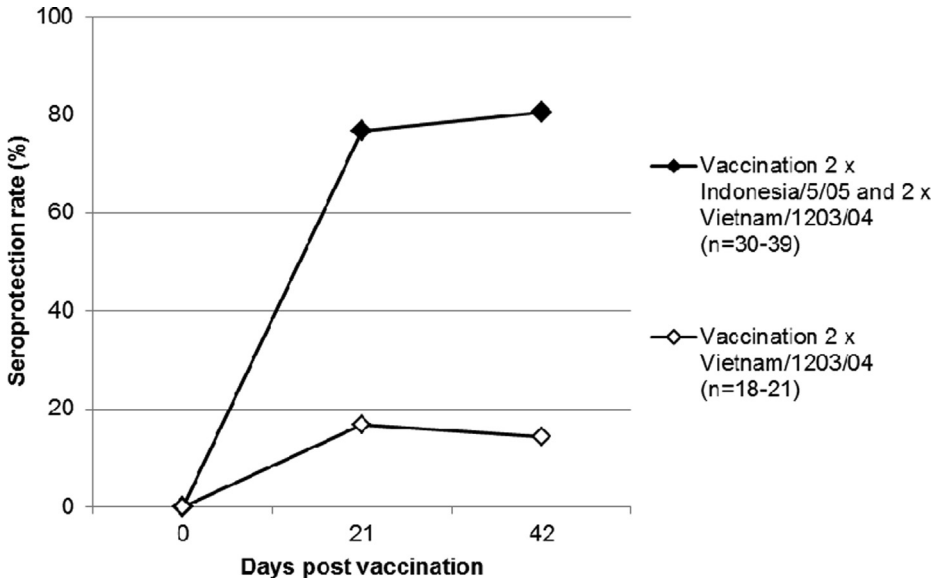

Fig. 3. Seroprotection rates against A/Vietnam/1203/2004 determined by haemagglutination inhibition (HI) test before and after vaccination of 60 subjects with two doses of non-adjuvanted A/Vietnam/1203/2004-like H5N1 vaccine (Baxter) in 2011. 21 subjects received their first A(H5N1) vaccination (Vietnam strain only; white markers) and 39 subjects had been immunized also earlier in 2009 (black markers) with two doses of AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine (GSK). Seroprotection rate was defined as the percentage of vaccinees with a haemagglutination inhibition titer ≥ 40.

MN testing was performed against ten different A(H5N1) candidate vaccine strains. All subjects studied by MN tests participated in both 2009 and 2011 vaccination trials. After the second dose in 2009 with AS03-adjuvanted A/Indonesia/5/2005 MN GMTs increased significantly (p < 0.01 – p < 0.0001) for all but that of the A/goose/Guiyang/ 337/2006 strain and the greatest fold increase 27.7 was logically seen against the homologous A/Indonesia/5/2005 vaccine strain (Table 2A&B). At days 182 and 730 GMTs were reduced yet the GMT value against the homologous vaccine virus strain (Indonesia) was significantly higher than before the vaccination.

A robust booster effect was seen when 2009 vaccinated subjects received two doses of non-adjuvanted A/Vietnam/1203/2004 vaccine in 2011. MN GMTs against all A(H5N1) viruses studied peaked at day 21 or 42 and stayed at significantly higher (p < 0.01 – p < 0.0001) levels compared to day 0 or day 730 after the booster vaccination (Table 2A&B). MN fold increases three weeks after the second vaccination (day 42) were 1.1–27.7 and 10.0–67.6 for the first vaccination in 2009 and booster vaccination in 2011, respectively. The very same booster effect was seen in those two individuals who had a history of MF59®-adjuvanted H5N1 pre-pandemic influenza vaccine 6–7 months before AS03-adjuvanted A/Indonesia/5/2005 vaccination in 2009. MN GMTs increased strongly depending on the viral strain to 40–2560 and 80–2560 at day 21 and day 42 after AS03-adjuvanted A/Indonesia/5/2005 vaccination, respectively (data not shown).

Next we analyzed the rate of seroprotection (SR) i.e. the percentage of individuals showing ≥ 1:80 titer in the MN test, before and after influenza vaccination. Three weeks after the second dose (day 42) with AS03-adjuvanted A/Indonesia/5/2005 in 2009 very high SR values (83.3%) against the homologous vaccine virus was seen (Fig. 5), whereas heterologous SRs ranged from 0 to 37.5% depending on the viral strain. After the booster vaccinations in 2011 MN SRs ranged from 45.8 to 87.5% to 52.0–92.0% for different virus strains at days 21 and 42, respectively. SRs remained elevated (40.9–68.2%) even two years after the booster vaccination for most of the viruses, while lower SR values were detected against the strains A/goose/Guiyang/337/2006 and A/chicken/Vietnam/NCVD-016/2008, which are genetically further away from the vaccine viruses (Fig. 2).

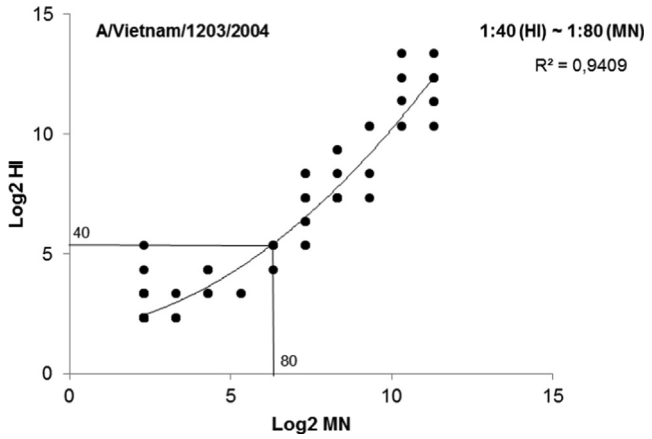

Fig. 4. Correlation of the antibody titers to A/Vietnam/1203/2004 measured by haemagglutination inhibition (HI) and microneutralization (MN) tests. Log-transformed HI and MN titers are presented, polynomial trend line and coefficient of determination calculated by linear regression analysis. Each dot may include the value from several serum specimens. Total number of serum specimens is 161.

The vaccination of humans against the influenza A(H5N1) virus with the pre-pandemic influenza A(H5N1) vaccine is usually comprised of two vaccine doses. To analyze the impact of multiple immunizations to heterologous response, we immunized rabbits and guinea pigs up to four times with the A/Indonesia/5/2005 vaccine. Analysis of the mean antibody responses in rabbits (Fig. 6A) indicates that the vaccine induces very high heterologous antibody levels in animals and the mean antibody levels reach their maximal or near-maximal levels after three immunizations. Additional immunization (4th dose) did not further increased the cross-reactivity of the antibodies against different A(H5N1) strains. Serum specimens from guinea pigs were collected only at day 0 before the first immunization and one week after the last vaccination (day 70). Mean antibody levels increased to very high levels after four doses of the vaccine (Fig. 6B). Day 70 HI-titers were throughout slightly higher in guinea pigs (GMTs 485–8914) compared to those of rabbits (GMTs 160–3378). The lowest cross-reactive antibody levels in guinea pig and rabbit anti-sera were observed for viruses A/goose/Guiyang/337/2006 and A/Vietnam/1203/2004.

Table 2
. A and B. Vaccine-induced humoral immune responses against different influenza A(H5N1) vaccine strains measured by microneutralization (MN) test. 25 subjects were vaccinated with two doses of AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine (GSK) in 2009 and non-adjuvanted A/Vietnam/1203/2004-like H5N1 vaccine (Baxter) in 2011. Day 0 refers to serum samples collected before the vaccination. Statistical differences between the pre-vaccine and post-vaccine antibody levels were calculated using Student's *t*-test. CI: confidence interval. Statistical comparison to day 0 GMT values,*p < 0.01 **p < 0.001 ***p < 0.0001.

Virus strain (clade)	**A/Vietnam/1203/2004** (1)		**A/duck/Hunan/795/2002 (2.1.1)**		**A/Indonesia/5/2005 (2.1.3.2)**		**A/whooper swan/ Mongolia/244/2005 (2.2)**		**A/Egypt/N03072/2010 (2.2.1)**	
Geometric mean titer [95% CI] *Fold increase*										
day 0 (n = 25)	5.0	[5.0–5.0]	5.0	[5.0–5.0]	5.0	[5.0–5.0]	5.0	[5.0–5.0]	5.1	[4.9–5.4]
day 21 (n = 21–22)	5.3*1.0*	[5.0–5.0]	5.3*1.1*	[4.7–6.1]	9.4*1.9*	[6.4–13.7] *	5.9*1.2*	[4.5–7.7]	6.4*1.3*	[4.5–9.2]
day 42 (n = 22–24)	7.3*1.5*	[5.8–9.2] *	16.0*3.2*	[10.7–24.0] ***	138.5*27.7*	[91.3–210.1] ***	25.7*5.1*	[17.7–37.4] ***	25.9*5.0*	[16.6–40.5] ***
day 182 (n = 22)	5.7*1.1*	[4.7–6.9]	8.0*1.6*	[6.0–10.7] *	28.3*5.7*	[19.6–40.8] ***	11.3*2.3*	[8.4–15.4] ***	13.7*2.7*	[8.9–21.2] **
day 0/730 (n = 25)	5.3	[4.9–5.7]	5.7	[4.9–6.7]	11.5	[8.2–16.0] ***	7.2	[5.6–9.2] *	8.2	[5.9–11.5]
day 21 (n = 24)	246.8*46.7*	[137.8–442.0] ***	190.3*33.1*	[100.9–359.0] ***	508.0*44.2*	[283.8–909.1] ***	261.4*36.5*	[142.2–480.7] ***	493.5*59.9*	[285.5–853.2] ***
day 42 (n = 25)	271.0*51.3*	[155.4–472.5] ***	183.8*32.0*	[100.1–337.4] ***	471.8*41.1*	[258.2–862.1] ***	278.6*38.9*	[151.0–514.0] ***	557.3*67.6*	[323.6–959.4] ***
day 182 (n = 25)	117.9*22.3*	[62.6–222.1] ***	97.1*16.9*	[48.7–193.8] ***	249.3*21.7*	[121.6–511.4] ***	143.2*20.0*	[71.4–287.2] ***	286.4*34.8*	[142.2–576.8] ***
day 365 (n = 24)	92.4*17.5*	[53.4–160.0] ***	71.3*12.4*	[36.6–139.0] ***	169.5*14.8*	[81.2–353.7] ***	106.8*14.9*	[49.2–231.9] ***	207.5*25.2*	[97.3–442.6] ***
day 730 (n = 22)	53.1*10.0*	[29.8–94.6] ***	38.8*6.7*	[22.1–68.0] ***	96.6*8.4*	[45.5–205.5] ***	58.4*8.1*	[30.1–113.1] ***	99.7*12.1*	[45.2–220.1] ***

Virus strain (clade)	**A/Egypt/3300-Namru3/ 2008 (2.2.1.1)**		**A/Hubei/1/2010 (2.3.2.1a)**		**A/Anhui/1/2005 (2.3.4)**		**A/goose/Guiyang/337/ 2006** (4)		**A/chicken/Vietnam/ NCVD-016/2008 (7.1)**	
Geometric mean titer [95% CI] *Fold increase*										
day 0 (n=25)	5.0	[5.0–5.0]	5.7	[4.7–7.0]	5.0	[5.0–5.0]	5.0	[5.0–5.0]	5.0	[5.0–5.0]
day 21 (n=21–22)	7.5*1.5*	[4.4–12.9]	7.7*1.3*	[5.3–11.1]	60.0*1.2*	[4.7–7.8]	5.0*1.0*	[5.0–5.0]	6.4*1.3*	[4.4–9.5]
day 42 (n=22–24)	47.6*9.5*	[26.5–85.4] ***	24.2*4.2*	[17.7–33.0] ***	19.4*3.9*	[13.3–28.3] ***	5.5*1.1*	[4.8–6.3]	11.6*2.3*	[7.8–17.3] **
day 182 (n=22)	18.2*3.6*	[11.1–29.8] ***	16.6*2.9*	[11.8–23.2] ***	11.0*2.2*	[7.8–15.4] **	5.2*1.0*	[4.9–5.5]	7.1*1.4*	[5.3–9.3]
day 0/730 (n=25)	9.2	[6.2–13.8] *	9.0	[6.5–12.3]	7.2	[5.7–9.1] *	5.0	[5.0–5.0]	5.6	[4.9–6.4]
day 21 (n=24)	479.5*52.1*	[261.6–878.9] ***	369.7*41.3*	[219.0–624.0] ***	302.0*42.1*	[179.0–509.6] ***	75.5*15.1*	[45.0–126.6] ***	56.6*10.1*	[27.1–118.1] ***
day 42 (n=25)	498.7*54.2*	[263.8–942.5] ***	399.5*44.6*	[226.0–706.0] ***	302.7*42.2*	[167.5–547.1] ***	62.3*12.5*	[35.6–109.1] ***	55.8*10.0*	[26.8–116.0] ***
day 182 (n=25)	294.5*32.0*	[129.7–668.6] ***	205.3*22.9*	[111.4–378.7] ***	143.2*20.0*	[73.4–279.5] ***	34.8*7.0*	[19.9–61.0] ***	34.8*6.2*	[17.2–70.5] ***
day 365 (n=24)	201.6*21.9*	[86.6–469.1] ***	155.4*17.4*	[86.0–281.1] ***	97.9*13.7*	[50.3–190.7] ***	27.5*5.5*	[16.9–44.6] ***	25.9*4.6*	[13.1–51.4] **
day 730 (n=22)	93.6*10.2*	[38.2–229.9] ***	87.9*9.8*	[49.9–155.1] ***	62.2*8.7*	[32.1–120.3] ***	17.6*3.5*	[11.0–28.2] ***	15.1*2.7*	[9.1–24.9] *

Statistical comparison to day 0 GMT values, *p < 0.01 **p < 0.001 ***p < 0.0001.

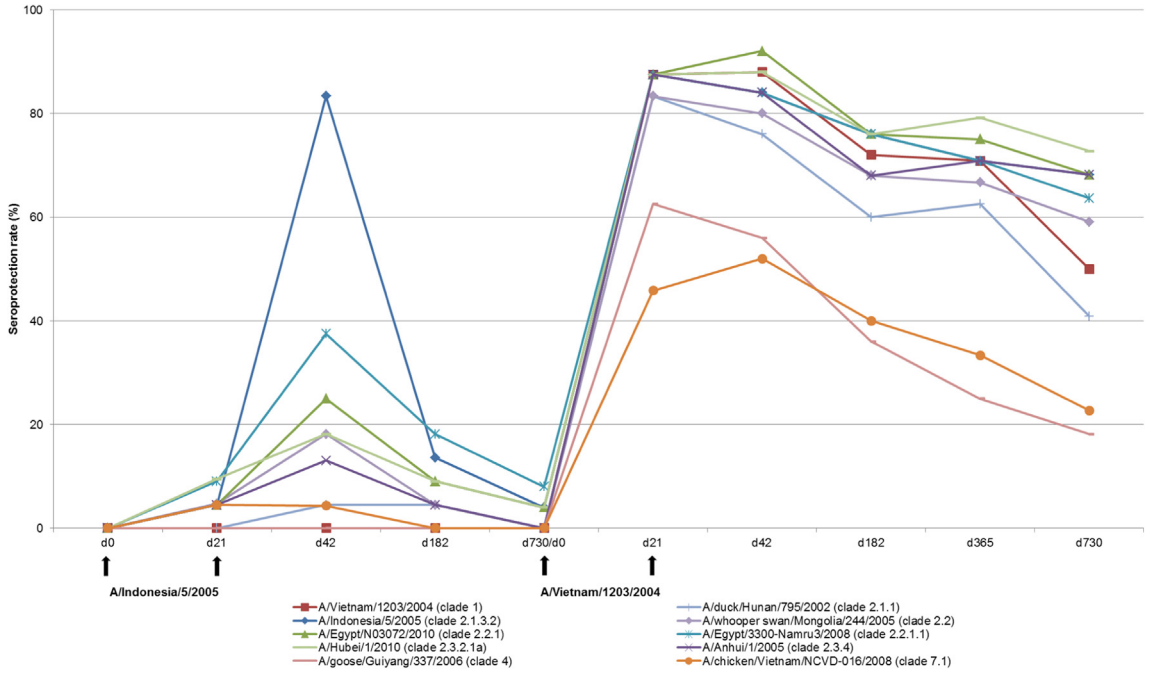

Fig. 5. Seroprotection rates determined by microneutralization (MN) test before and after vaccination of 25 subjects who received two doses of AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine (GSK) in 2009 and two doses of non-adjuvanted A/Vietnam/1203/2004-like H5N1 vaccine (Baxter) in 2011. Seroprotection rate was defined as the percentage of participants with a microneutralization titer $\geq$ 80.

4. Discussion

The spread of a new HPAI A(H5N1) virus across Asia in 2004 infecting both poultry and people raised the concerns for a new pandemic threat. WHO started to actively follow-up the epidemiology of avian influenza viruses and increased their activities in avian and pandemic influenza preparedness [43]. To date, the transmissibility of the A(H5N1) viruses in humans has remained limited yet the pandemic potential of the A(H7N9) viruses may be greater than that of the currently circulating HPAI A(H5N1) viruses [44]. However, the first influenza pandemic of the 21st century was caused by a novel reassortant A(H1N1) swine influenza virus with gene segments originating from avian, human and swine viruses in 2009 [45]. Surprisingly, the next pandemic was not caused by an influenza A virus but by a coronavirus, severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) causing the coronavirus disease (COVID-19) that started to spread throughout the world in 2020 [46].

WHO coordinates the development of influenza candidate vaccine viruses (CVVs) for pandemic preparedness. National authorities may consider to use one or more of these CVVs for example in clinical trials and other pandemic preparedness purposes based on their assessment of public health risk and need [5]. During the year 2006 the health officials in Finland purchased an avian influenza vaccine prototype sufficient to vaccinate all residents of the country. The vaccines were received in 2008 and they would have been used if the HPAI A(H5N1) strain of avian influenza virus had been causing a pandemic in humans. An advance reservation contract was also made to buy a more targeted avian influenza vaccine developed in a potential pandemic situation.

In the present study we first analyzed the immunogenicity of the Finnish pandemic stockpile vaccine in humans and in animals. As previously suggested [47–48], two doses of inactivated, AS03-adjuvanted A/Indonesia/5/2005-like split virion A(H5N1) vaccine was administered to volunteers in 2009. After the second vaccination MN GMTs increased significantly against all but the A/goose/Guiyang/ 337/2006 strain and the greatest fold increase and seroprotection rate was logically seen against the homologous vaccine strain. Antibody responses to heterologous strains were much lower than to the homologous vaccine strain and varied according to the virus strain tested. The two subjects who had received two doses of MF59®-adjuvanted A/Vietnam/1194/2004 H5N1 vaccine (Novartis) 6–7 months prior to A/Indonesia/5/2005 vaccine showed very strong heterologous antibody responses.

To analyze whether multiple immunizations would enhance vaccine-induced antibody levels and broaden the cross-reactivity against different vaccine A(H5N1) virus strains, we immunized rabbits and guinea pigs up to four times with AS03-adjuvanted A/Indonesia/5/2005 vaccine. Very high heterologous antibody levels were detected after four immunizations, yet rabbit antibody responses suggested that three vaccine doses were sufficient to induce maximal antibody levels in vaccinated animals. Higher antibody titers observed in guinea pigs may reflect differences between animal species and also indicate reported dose–response effect [33] as compared to size immunized animals. Heterologous antibody responses in rabbits and guinea pigs were in line with the observations in humans showing the lowest cross-reactive antibodies to A/goose/Guiyang/337/2006 and A/Vietnam/1203/2004 viruses which are phylogenetically distinct from the Indonesian vaccine virus clade.

Different antigen doses, adjuvants and prime-boosting strategies have been used in A(H5N1) vaccine studies in humans. Two doses of A(H5N1) vaccine was required to fulfill the licensure criteria for the homologous response whether the doses were given 21 days or 6 months apart [49]. AS03-adjuvanted vaccine generally resulted in the strongest cross-clade responses with the highest vaccine antigen dose used [33]. The AS03-adjuvanted A/Indonesia/05/2005 vaccine was more immunogenic compared to non-adjuvanted vaccine in homologous assays [32] and priming with a single dose of AS03-adjuvanted A/Indonesia/05/2005 vaccine induced rapid and durable antibody responses to a heterologous AS03-adjuvanted A/turkey/Turkey/1/2005 booster vaccination 6

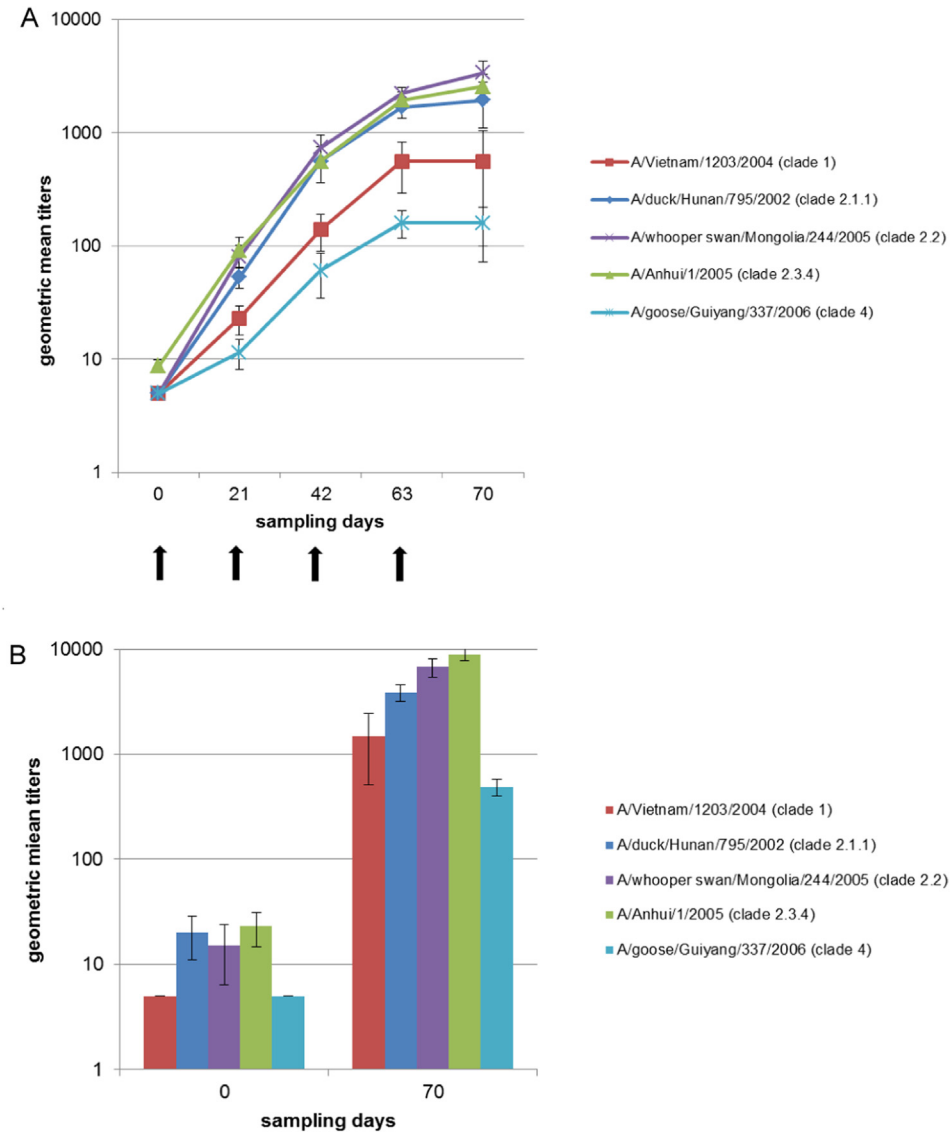

Fig. 6. Geometric mean titers of anti-A(H5N1) virus antibodies in rabbits and guinea pigs receiving multiple doses of AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine (GSK). Rabbits (A) and guinea pigs (B) were immunized for four times at three week intervals with AS03-adjuvanted A/Indonesia/5/2005-like H5N1 vaccine and the serum samples were collected on day 0 (rabbits and guinea pigs), before each immunization (rabbits only), and one week after the last immunization (rabbits and guinea pigs). The immunizations are marked with arrows. The serum samples were analyzed by haemagglutination inhibition (HI) test and geometric mean titers for each virus were calculated. The means represent the geometric means of five rabbit and five guinea pig anti-sera.

or 18 months later [23]. Priming with a non-adjuvanted high dose A/Vietnam/1203/2004 vaccine boosted by a single low dose of MF59-adjuvanted A/Anhui/1/2005 vaccine 19–25 months later generated moderate cross-reactive antibody responses whereas this was not the case with homologous prime-boost [50].

One study performed by cytopathic effect-based MN assays with a lower seroprotection threshold of 1:20 suggested a significant homologous antibody rise after two doses of non-adjuvanted whole-virus A/Vietnam/1203/2004 vaccine [20]. In contrast to that study, we detected for the same vaccine virus only weak homologous seroprotection rates after one or two vaccine doses in naïve seronegative subjects. However, our criteria for seroprotection was based on MN titer of 1:80 and thus different assays and seroprotection correlates may provide different interpretation of the data. However, we demonstrated that the non-adjuvanted A/Vietnam/1203/2004 vaccine induced robust booster effect and heterologous response for subjects primed with AS03-adjuvanted A/Indonesia/05/2005 vaccine two years earlier. Priming with one or two primary doses AS03-adjuvanted A(H5N1) vaccine has been shown to give rise to heterologous AS03-adjuvanted boosting after 12 months [16]. Consistent with our data the highest cross-reactive humoral (and CD4 + cell-mediated) immune responses were observed when two primary doses were followed by a heterologous booster vaccine [16]. The booster effect has been suggested to be the highest two years after two dose priming compared the booster response seen at six or 12–15 months after primary immunization [20]. Our results further confirm earlier observations that heterologous prime-boost vaccination induces more cross-reactive responses which may give rise to a much broader protection against novel A(H5N1) virus strains [35,50].

Cross-protective neutralizing antibodies in patients severely infected by A(H5N1) viruses were shown to persist longer than 2 years while those of asymptomatically infected individuals showed the disappearance of neutralizing antibodies within a year [51]. Only a few studies have observed the persistence of cross-reactive antibodies longer than one year after a heterologous A

(H5N1) booster vaccination [23]. We demonstrated that vaccine-induced humoral immune responses against all viruses studied were significantly higher even two years after the non-adjuvanted booster vaccination. SRs for all viruses waned but stayed at clearly elevated levels except against the phylogenetically more distinct strains A/goose/Guiyang/337/2006 and A/chicken/Vietnam/NCVD-016/2008.

Neutralizing A(H5N1) antibody responses have usually confirmed the trend seen in the HI test [16,33,35,49]. However, the correlation has been lower for A/Vietnam/1194/2004 and A/Hubei/1/2020 strains [33]. We observed that the antibody titers of the HI and ELISA-based MN assay correlated strongly positively and defined that the A/Vietnam/1203/2004 HI titer of 40 corresponded roughly to a MN titer of 80. It is also noteworthy that MN titer of 80 has been defined as a serological marker of H5N1 infection [52]. However, the data on the protective value of MN antibody titers against influenza varies considerably depending on the viruses and method used in the assays [53–55]. Also $\geq$ 4-fold increase in MN titers relative to the baseline value has been used as a correlate of protection [32]. The MN assays appear to have a greater sensitivity [53,55–56] than the HI assay, particularly in serum specimens that show low anti-viral antibody levels [57]. Although MN assays have displayed more intra- and interlaboratory variability than the HI assay [57], a good correlation has been shown between the results of different MN assays [58]. A special advantage has been seen in ELISA-based neutralization assays since they tend to show lower variation [57].

Our study has certain limitations. Firstly, as the number of subjects participating was no larger than 61–64 and all age groups were not covered, the results do not necessarily apply to elderly or children. The immunogenicity of vaccines in general remains lower among the elderly than among children and adults [59]. Two dose A(H5N1) priming has been shown to be equally immunogenic in adults and the elderly [19–20], but among the elderly the magnitude and duration of heterotypic T cell responses have remained lower [29]. In infants and young children heterologous prime-boost vaccination antibody responses to homologous and heterologous vaccine strains has shown to persist at least six months following the booster vaccination [60]. Secondly, the results obtained in our two vaccination scheme are not comparable as such, since the vaccines were not administered during the same year. Thirdly, in the present study we only analyzed humoral immune responses and did not measure cell-mediated immune responses. In previous studies analysis of T cell responses have shown similar heterotypic HA prime-boosting responses as those seen in humoral responses [16,29].

In the present study we optimized and used an MN assay together with a traditional HI assay to investigate antibody responses induced by two different pre-pandemic influenza A (H5N1) virus vaccines. We demonstrated high homologous and heterologous antibody responses against different genetic clades of influenza A(H5N1) viruses after heterologous booster vaccination. The results suggest strong long-lasting vaccine-induced cross-clade immunity against influenza A(H5N1) strains after booster vaccination, which may broaden the antibody responses to emerging viruses. Our data provides further evidence that priming individuals with one type of pre-pandemic H5N1 avian influenza vaccine prepares the person to a much stronger heterotypic response induced by another vaccine type. This observation may justify the rationale of the use of pre-pandemic vaccines.

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.

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Authors' contributions

All authors attest they meet the ICMJE criteria for authorship. AH performed MN tests, the serological data analysis and wrote the manuscript. NI made the phylogenetic tree. NI, CSK and IJ also participated in the writing of the manuscript. IJ was involved in the design of vaccination study and sera collections. All authors have read and approved the final version of the manuscript.

Ethics statement

The samples obtained were part of a study conducted at the Finnish Institute for Health and Welfare (THL), Finnish Food Authority and Helsinki University Hospital. Written informed consent was obtained from all participants. The study protocol and consent was approved by the Ethics Committee of the Department of Medicine, Helsinki University Hospital (Permission 250/13/03/00/2011).

Role of the funding source

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