ORIGINAL ARTICLE

The Madin-Darby canine kidney cell culture derived influenza A/H5N1 vaccine: A Phase I trial in Taiwan

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Background: Avian H5N1 influenza has caused human infections globally and has a high mortality rate. Rapid production of effective vaccines is needed.
Methods: A phase 1, randomized, observer-blinded clinical trial was conducted to examine the safety and immunogenicity of an inactivated whole virion vaccine against the influenza A/H5N1 virus produced from the Madin-Darby canine kidney (MDCK) cell line. Participants were randomized to four groups and administered two intramuscular doses of vaccine containing 3 μg hemagglutinin (HA), 3 μg HA with 300 μg aluminum phosphate ($AlPO_4$), 6 μg HA, and 6 μg HA with 300 μg $AlPO_4$, respectively, at two visits, 21 days apart. Serum hemagglutination inhibition (HAI) and neutralizing antibody levels were determined at baseline and on Days 21 and 42.
Results: Sixty healthy individuals were enrolled. The neutralization assay showed a significant immune response in the 6 μg with $ALPO_4$ group on Day 42 compared to pre-vaccination levels (11.32 ± 9.77 vs. 4.00 ± 0, $p = 0.02$). The adjuvant effect in neutralization assay was also significant on Day 42 in the 6 μg group (4.52 ± 1.94 without adjuvant vs. 11.32 ± 9.77 with adjuvant, $p = 0.02$). HAI assay also showed an aluminum adjuvant-induced increasing trend in HAI geometric mean titer on Day 42 in the 3 μg and 6 μg groups (6.02 versus 8.20, $p = 0.05$ and 5.74 versus 8.21, $p = 0.14$). The most frequent adverse event was local pain (20% to 60%). There were no vaccine-related severe adverse effects.

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Conclusion: MDCK cell line-derived H5N1 vaccine was well tolerated. It is necessary to investigate further the immunogenicity of higher antigen doses and the role of aluminum adjuvant in augmenting the effect of the vaccine.

Introduction

Avian H5N1 influenza has been infecting humans globally since 1997 and has a high mortality rate. According to the bulletin issued by the World Health Organization, 607 human infections and 358 deaths have been attributed to H5N1 infection up to July 2012 in Africa, Asia, and Europe.[1] Experts warn that the next influenza pandemic is imminent and could be severe. Prevention and control will depend on the rapid production and worldwide distribution of specific pandemic influenza candidate vaccines.

A number of challenges have been encountered during the preparation of pandemic influenza vaccines. Firstly, timely isolation of the strain and mass production are very important; secondly, a two-dose vaccination protocol has been recommended in order to achieve protective antibody levels in immunologically naive vaccine recipients.[2–5] Rapid production of adequate amounts of H5N1 HA antigen for vaccination preparation is therefore an important challenge.

Although the HA antigen has been traditionally produced in eggs, an H5N1 influenza vaccine was recently successfully produced from whole virus grown in Madin-Darby canine kidney (MDCK) cells.[6] This approach used a continuous cell line production for the rapid supply for HA antigen and circumvented the risk of shortage of egg-culture derived vaccine during avian influenza epidemics.[7,8] MDCK-derived purified inactivated H5N1 vaccine antigens were safe and induced immune responses in animal studies.[6] Moreover, formulation with adjuvants such as aluminum phosphate elicited a stronger response in animals, even at low doses.[9] However, it is necessary to evaluate the safety and immunogenicity of these vaccines in human studies. In this human Phase I clinical study, we evaluated the safety and immunogenicity of nonadjuvant and alum formulated H5N1 virion influenza vaccine derived from MDCK cells.

Materials and methods

Study design

The main objective of this prospective, randomized, open label, observer-blind, single-center study, was to evaluate the safety, reactogenicity and humoral immune responses to inactivated H5N1 influenza virion vaccines, either in the nonadjuvanted form or formulated with aluminum phosphate (AlPO$_4$). The study recruited a total of 60 healthy individuals at the National Taiwan University Hospital between November 25, 2009 and January 7, 2011. Study participants were randomized to 4 groups, as follows: Group I received 3 μg haemagglutinin (HA); Group II received 3 μg HA with adjuvant (300 μg AlPO$_4$); Group III

received 6 μg HA; and Group IV received 6 μg HA with adjuvant (300 μg AlPO$_4$). The participants were administered two doses of 0.5 ml H5N1 vaccine, on Days 0 and 21.

Recruitment of study participants was carried out in two parts. In Part 1, the initial 12 participants were sequentially enrolled and randomized to three blocks; Block A, Block B and Block C. The first four participants of Block A received two doses of vaccination, 21 days apart, and local/systemic reactions and adverse events were recorded after each vaccination. Block B participants were then enrolled and received their vaccinations 7 days after Block A participants had received their second vaccine. Block C participants were enrolled and received their vaccinations 7 days after the Block B participants had received their second vaccine. The safety data and adverse events (recorded on diary cards) were reviewed after vaccine administration. After it was determined that there were no safety concerns with the vaccine, the rest of the study participants (48 participants) were recruited in Part 2 of the study.

The study followed the principles of the Declaration of Helsinki, Good Clinical Practice (as defined by the International Conference on Harmonisation), and Taiwanese regulatory requirements. The study protocol was approved by the human research ethics committee in National Taiwan University Hospital and all study participants provided written informed consent.

Participants

This study recruited healthy male and female individuals (age ≥20 and ≤60 years at the time of enrolment). The female participants were: (1) of nonchildbearing potential, i.e. surgically sterilized (defined as having undergone hysterectomy and/or bilateral oophorectomy and/or bilateral salpingectomy); (2) 1 year postmenopausal; or (3) had agreed to practice abstinence or use adequate contraceptive precautions for 30 days prior to the study and for 2 months after study completion. Female participants were also required to have a negative pregnancy test and to not be breastfeeding for the duration of the study.

Exclusion criteria included: (1) previous known or potential exposure to avian influenza virus or any adjuvanted or unadjuvanted H5N1 HA antigen vaccine; (2) vaccination with any influenza vaccine within 6 months prior to study enrolment or at any time during the study period; (3) presence of confirmed or suspected abnormal immune function, immunosuppressive, or immunodeficient condition, including human immunodeficiency virus infection; (4) history of hypersensitivity to vaccines or a history of allergic disease or reactions likely to be exacerbated by any component of the vaccine; (5) chronic administration (defined as >14 days) of immunosuppressants or other

immune-modifying drugs within 6 months prior to the administration of the study vaccine (corticosteroids, including prednisone or equivalent, $\geq$ 0.5 mg/kg/day; inhaled and topical steroids were allowed); (6) presence of any medical illness including clinically significant acute pulmonary, cardiovascular, hepatic, or renal functional abnormality, as determined by physical examination or laboratory screening tests; (7) administration of immuno-globulins and/or any blood products within the three months preceding the administration of the study vaccine or at any time during the study; (8) presence of acute disease at the time of enrolment (defined as the presence of a moderate or severe illness with or without fever/oral temperature $\geq 37.5°C$); (9) presence of a fever (oral temperature $\geq 37.5°C$) at the time of enrolment; (10) aspartate aminotransferase, alanine aminotransferase, or serum creatinine ≥ 1.5 times upper limit normal value; (11) complete blood count deemed unsuitable for vaccination by investigator; (12) chronic or long-term use of acetylsalicylic acid medication; (13) participation in other clinical studies of investigational drugs within 3 months prior to the start of the present study; or (14) participation in other clinical studies of investigational vaccines within 6 months prior to the start of this study.

Vaccine

The monovalent vaccine seed virus was an H5N1 reassortant reference virus (A/Vietnam/1194/2004, NIBRG-14) derived by reverse genetics from the highly pathogenic avian strain A/Vietnam/1194/2004 (wild type) by the UK National Institute for Biological Standards and Control. The seed virus was cultivated in eggs for 4 generations at the Centers for Diseases Control, Taiwan, and then transferred to the Vaccine Research and Development Center of the National Health Research Institution, Taiwan for a further 5-passage adaptation in MDCK cells. MDCK-grown, sucrose-gradient purified, and formaldehyde-inactivated whole virus candidate vaccine was developed and produced by Vaccine Research and Development Center.[6,9]

The study vaccine containing inactivated whole influenza virus and 0.01% formaldehyde, was formulated in phosphate buffered saline (PBS). The vaccine (7 mL liquid/ vial) was produced as a multidose formula in 20 mL vials (0.5 mL/dose, total 14 doses/vial; each vial applied to 10 participants). Different formulations of the vaccine were made using two different strengths of HA protein, in non-adjuvant or AlPO4 formulated. All vaccines to be administrated to participants were stored at the defined temperature range ($+2°C$ to $+8°C$) in a safe and locked place. The storage unit was monitored by a continuous temperature monitoring device.

Formulated vaccine, in a volume of 0.5 ml as a single dose, was withdrawn for administration to the participants. Intramuscular administration of the vaccine was done in the deltoid region of the nondominant arm.

Safety

All study participants were monitored for self-reported as well as investigator-assessed local and general adverse events during the 6 weeks following the first vaccination. Complete blood count, serum biochemistry, adverse events, and serious adverse events (SAEs) were evaluated.

Immunogenicity

Serum samples were obtained prior to vaccination, as well as on Days 21 and 42 after vaccination. Antibody titers were measured by hemagglutination-inhibition (HAI) assays and virus neutralization assays. Based on international guidelines, three immunogenicity end-points were applied to evaluate the different formulations of the influenza vaccines.[10,11] These included: (1) the seroprotective rate (the proportion of participants with antibody level $\geq 1:40$ on HAI assay); (2) the seroconversion rate (the proportion of participants with a prevaccination HAI antibody titer $<1:10$ and a post-vaccination titer $\geq 1:40$, or a prevaccination titer $\geq 1:10$ and an increase in the titer by a factor of four or more); and (3) evaluation of the geometric mean titers (GMT) of the HAI antibody titer.

Microneutralization assays were performed according to previous reports.[6,9] Thawed human serum samples were used without further treatment in the assay. Each serum was tested in quadruplicate.

Statistical analyses

To compare the characteristics between the four dosing groups, the ANOVA test was applied for continuous variables and Fisher's exact test was used for categorical variables. Wilcoxon rank sum test was applied for the comparison between either 2 dosing groups for continuous variables and fisher's exact test for categorical variables. Wilcoxon signed rank test was used for comparison again the pre-post vaccination level of titers. The seroprotection and the seroconversion rates were summarized by rate and 95% confidence intervals. The 95% confidence intervals of the geometric mean titers were obtained by taking anti-log for the statistics of the log titer. All statistical analyses were performed by SAS software version 9.2 (SAS, Cary, NC, USA) and all tests were two-sided when applied. Statistical significance for all comparisons was determined at $p < 0.05$.

Results

Study participants

Of the 61 adult individuals recruited in this study, one had elevated liver function markers (defined as aspartate aminotransferase or alanine aminotransferase ≥ 1.5 times the upper limit normal value) prior to the study and was therefore excluded. The remaining 60 participants were randomly divided into 4 groups as described in Fig. 1. The demographic characteristics of all vaccinated participants are summarized in Table 1. Of the two participants who did not receive the second dose of the vaccine, one failed to come in within the acceptable time window for the second dose and the second had elevated liver function tests after the first dose of the vaccine. These two participants were
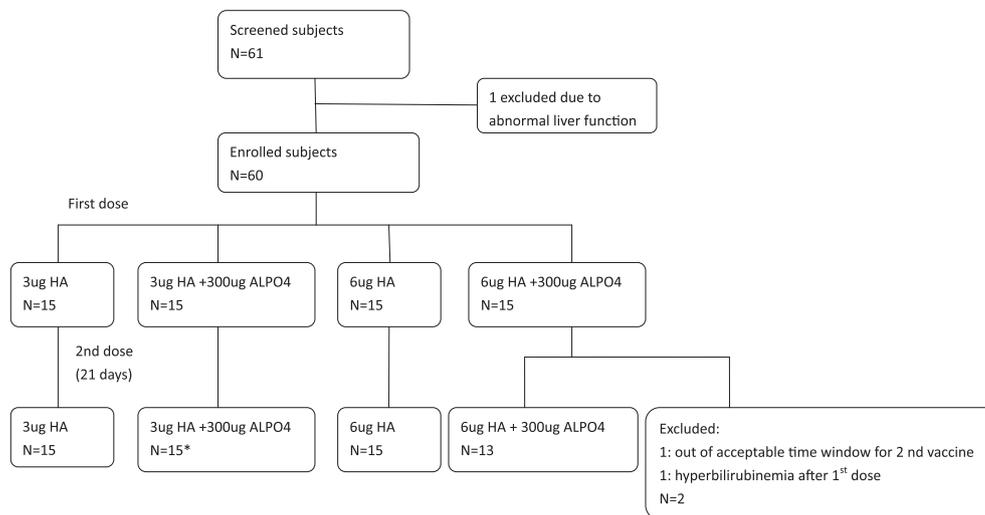
Figure 1. Enrollment and follow up of the study participants. *One participant was excluded from immunogenicity analysis, even though they received two doses of vaccination, due to receiving another investigational vaccine.

therefore excluded from the immunogenicity analysis. A third participant received a second investigational vaccine 125 days after the second dose of vaccine in our study and was therefore also excluded from the immunogenicity analysis. Our safety analyses therefore included a total of 60 participants, while the immunogenicity analyses included 57 participants.

Safety

Table 2 summarizes the injection-site and systemic reactions that occurred during the 7 days following each vaccination. Our data showed that 38 participants (32.2%) had at least one solicited local event, while 30 participants (25.4%) had at least one solicited systemic event. The most frequently reported symptom among all the groups was

local pain (20% to 60%). Symptoms remained mostly mild to moderate in intensity and resolved rapidly. The groups receiving adjuvanted vaccines did not have a significantly higher occurrence of solicited symptoms. We also found no antigen-dose effect on the reactogenicity of the candidate vaccine.

Data showed that 35 participants (29.7%) reported unsolicited adverse events after administration of vaccination. The most common reported unsolicited events included upper respiratory tract infection in 6 participants (5%) and dizziness in 8 (6.7%). Five participants had elevated liver function test after the first dose of vaccination and 2 after the second dose of vaccination. The intensity of abnormal liver function was mild to moderate. One participant developed hyperbilirubinemia after the first dose (total bilirubin was elevated from 1.5 mg/dL to

Table 1 Demographic characters of all vaccinated participants

	3 μg HA $n = 15$	3 μg HA with AlPO$_4$ $n = 15$	6 μg HA $n = 15$	6 μg HA with AlPO$_4$ $n = 15$	P value
Sex, no (%)					0.54
Male	3 (20.0%)	4 (26.7%)	4 (26.7%)	7 (46.7%)	
Female	12 (80.0%)	11 (73.3%)	11 (73.3%)	8 (53.3%)	
Age, years					0.66
Mean ± SD	32.0 ± 7.5	29.4 ± 8.8	28.5 ± 10.9	28.7 ± 6.5	
Range	20.2—47.3	20.1—48.8	20.1—48.9	20.4—42.0	
Race, no (%)					1.00
Chinese	15 (100.0%)	15 (100.0%)	15 (100.0%)	14 (93.3%)	
Asian, other than Chinese	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.7%)	
Medical history, no (%)					0.07
Yes	10 (66.7%)	5 (33.3%)	11 (73.3%)	11 (73.3%)	
No	5 (33.3%)	10 (66.7%)	4 (26.7%)	4 (26.7%)	
Concomitant medication					0.24
Yes	5 (33.3%)	3 (20.0%)	8 (53.3%)	7 (46.7%)	
No	10 (66.7%)	12 (80.0%)	7 (46.7%)	8 (53.3%)	

AlPO$_4$: aluminum phosphate; HA = hemagglutinin.

Table 2 Proportion of participants having a solicited local or systemic event within 7 days after each vaccination

	3 μg HA	3 μg HA with AlPO$_4$	6 μg HA	6 μg HA with AlPO$_4$
First dose	$n = 15$	$n = 15$	$n = 15$	$n = 15$
Any solicited local event	3 (20.0%)	6 (40.0%)	6 (40.0%)	9 (60.0%)
Hematoma	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Pain	3 (20.0%)	6 (40.0%)	6 (40.0%)	9 (60.0%)
Redness	1 (6.7%)	3 (20.0%)	0 (0.0%)	1 (6.7%)
Swelling	1 (6.7%)	2 (13.3%)	1 (6.7%)	0 (0.0%)
Second dose	$n = 15$	$n = 15$	$n = 15$	$n = 13$
Any solicited local event	4 (26.7%)	4 (26.7%)	4 (26.7%)	2 (15.4%)
Hematoma	1 (6.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Pain	4 (26.7%)	4 (26.7%)	4 (26.7%)	2 (15.4%)
Redness	1 (6.7%)	2 (13.3%)	0 (0.0%)	0 (0.0%)
Swelling	1 (6.7%)	0 (0.0%)	1 (6.7%)	0 (0.0%)
First dose	$n = 15$	$n = 15$	$n = 15$	$n = 15$
Any solicited systemic event	4 (26.7%)	3 (20.0%)	3 (20.0%)	5 (33.3%)
Fatigue	2 (13.3%)	2 (13.3%)	2 (13.3%)	3 (20.0%)
Fever	0 (0.0%)	0 (0.0%)	0 (0.0%)	1 (6.7%)
Headache	2 (13.3%)	0 (0.0%)	2 (13.3%)	1 (6.7%)
Joint pain	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Muscle ache	3 (20.0%)	2 (13.3%)	2 (13.3%)	4 (26.7%)
Shivering	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Second dose	$n = 15$	$n = 15$	$n = 15$	$n = 13$
Any solicited systemic event	5 (33.3%)	4 (26.7%)	4 (26.7%)	2 (15.4%)
Fatigue	3 (20.0%)	2 (13.3%)	2 (13.3%)	1 (7.7%)
Fever	1 (6.7%)	0 (0.0%)	0 (0.0%)	0 (0.0%)
Headache	3 (20.0%)	2 (13.3%)	1 (6.7%)	0 (0.0%)
Joint pain	0 (0.0%)	0 (0.0%)	1 (6.7%)	0 (0.0%)
Muscle ache	2 (13.3%)	1 (6.7%)	1 (6.7%)	1 (7.7%)
Shivering	0 (0.0%)	0 (0.0%)	0 (0.0%)	0 (0.0%)

2.0 mg/dL 21 days after the first dose) and therefore did not receive the second dose of vaccination. There were no deaths reported before the release of this report. There were also no SAEs during the study period.

Immunogenicity assessment

HAI assays and neutralization assays were used to measure the humoral immune response against the vaccine strain. Immunogenicity data were also evaluated in the pre-protocol cohort.

HAI assays

Prior to the vaccination, none of the study participants had protective antibody titers ($\geq$1:40). There was no significant difference in the seroprotection rate between the groups at 21 days and 42 days after the vaccination (Table 3).

There was also no significant difference in the seroconversion rate between the different dosing groups. The

seroconversion rate among all study participants was 4/57 (7.0%) on Day 21 (after the first vaccination) and 5/57 (8.8%) on Day 42 (after the second vaccination).

The HAI GMT and the mean fold rise against H5N1 virus are summarized in Table 3. There was no significant increase in the mean fold increase of GMT in the 4 groups at 21 days and 42 days after vaccination. There was also no significant dosing response. However, there was a trend of higher HAI GMT in groups that received adjuvant compared to groups that did not receive adjuvant at 42 days after vaccination, both in the 3 μg group ($p = 0.05$) and 6 μg group ($p = 0.14$).

Neutralization assays

One sample from the participant in the 6 μg group was retested and defined as an outlier, based on the laboratory data (neutralization assay was 126.49 on Day 42 while the mean was 12.65 ± 31.55) and was therefore excluded from the neutralization assay. Of the remaining 56 cases analyzed, there was a significant increase in neutralization assay values in the 6 μg with ALPO$_4$ group on Day 42 compared to pre-vaccination values (11.32 ± 9.77 vs. 4.00 ± 0, $p = 0.02$). An adjuvant effect was also noted in the 6 μg group on Day 42 (4.52 ± 1.94 without adjuvant vs. 11.32 ± 9.77 with adjuvant, $p = 0.02$; Table 3).

Discussion

In this study, we showed that the candidate H5N1 vaccine from MDCK cell line was well tolerated and no SAEs were noted at the dosing range used. Results from the neutralization assay showed a significant immune response in the 6 μg with adjuvant group, while no significant response was noted in the seroprotection rate, seroconversion rate and HAI GMT. The neutralization assay also showed an adjuvant effect with ALPO$_4$ in the 6 μg group, and this trend was also noted in the HAI GMT in the 3 μg as well as 6 μg groups.

Vaccination has been shown to be the major pathway to preventing influenza epidemics. However, in the case of novel influenza strains such as H5N1, vaccine production faces the challenges as timely requirement for strain isolation and mass production for the mostly naïve population. The traditional egg-based culture vaccine manufacturing methods, which are labour-intensive and lack flexibility, may therefore be inadequate to meet production demands in an influenza pandemic. Importantly, in an avian pandemic, the supply of embryonated eggs may be threatened, since avian influenza also infects egg-producing poultry.

There has been a recent focus on strategies for egg-independent vaccine production. Reverse genetics-based methods have been used to create attenuated strains by removing the polybasic amino acid sequence responsible for virulence. However, the antigen yield from reverse genetics-derived H5N1 viruses constituted only 30% to 40% of the average of seasonal influenza viruses.[12] Vaccine production based on cell cultures has shown promise and offers the advantage of flexibility for rapid scale-up, reduced risk for contamination in closed scalable bioreactors, and does not depend upon a supply of embryonated

Table 3 Immune response among participants receiving different dose, with/without adjuvant of study vaccine

	(a)3 μg HA $n = 15$	(b)3 μg + AlPO$_4$ $n = 14$	p^a	(c)6 μg HA $n = 15$	(d)6 μg + AlPO$_4$ $n = 13$	p^b
HAI seroprotection (titer ≥1:40) % (95% CI)						
Prevaccination	0.00% (0.00%, 0.00%)	0.00% (0.00%, 0.00%)	—	0.00% (0.00%, 0.00%)	0.00% (0.00%, 0.00%)	—
21 days	0.00% (0.00%, 0.00%)	7.14% (0.18%, 33.87%)	0.48	6.67% (0.17%, 31.95%)	15.38% (1.92%, 45.45%)	0.58
42 days	6.67% (0.17%, 31.95%)	7.14% (0.18%, 33.87%)	1.00	6.67% (0.17%, 31.95%)	15.38% (1.92%, 45.45%)	0.58
HAI seroconversion rate (%) (95% CI)						
21 days	0.00% (0.00%, 0.00%)	7.14% (0.18%, 33.87%)	0.48	6.67% (0.17%, 31.95%)	15.38% (1.92%, 45.45%)	0.58
42 days	6.67% (0.17%, 31.95%)	7.14% (0.18%, 33.87%)	1.00	6.67% (0.17%, 31.95%)	15.38% (1.92%, 45.45%)	0.58
HAI geometric mean titer (95% CI)						
Prevaccination	5 (5.00, 5.00)	5 (5.00, 5.00)	1.00	5 (5.00, 5.00)	5 (5.00, 5.00)	1.00
21 days	5 (5.00, 5.00)	6.10 (3.97, 9.35)	0.34	5.74 (4.27, 7.73)	6.89 (4.30, 11.04)	0.50
42 days	6.02 (4.05, 8.94)	8.20 (5.20, 12.94)	0.05	5.74 (4.27, 7.73)	8.52 (5.08, 14.30)	0.14
	(a)3 μg HA $n = 15$	(b)3 + AlPO$_4$ $n = 14$	p^a	(c)6 μg HA $n = 14^c$	(d)6 + AlPO$_4$ $n = 13$	p^b
Neutralization assays (95% CI)						
Prevaccination	6.02 ± 6.12 (2.63, 9.41)	4.52 ± 1.94 (3.40, 5.64)	0.60	4.00 ± 0 (4.00, 4.00)	4.00 ± 0 (4.00, 4.00)	1.00
21 days	6.57 ± 8.60 (1.81, 11.34)	5.45 ± 3.04 (3.70, 7.20)	0.65	4.38 ± 1.43 (3.56, 5.21)	8.28 ± 11.35 (1.42, 15.13)	0.24
42 days	6.43 ± 7.36 (2.35, 10.50)	8.64 ± 8.32 (3.83, 13.44)	0.36	4.52 ± 1.94 (3.40, 5.64)	11.32 ± 9.77 (5.41, 17.23)	0.02
Neutralization assays geometric mean titer (95% CI)						
Prevaccination	2.67 (1.73, 4.11)	2.38 (1.80, 3.15)	1.00	2.00 (2.00, 2.00)	2.00 (2.00, 2.00)	1.00
21 days	2.69 (1.70, 4.26)	2.86 (1.89, 4.32)	0.65	2.47 (1.89, 3.21)	3.32 (1.80, 6.12)	0.72
42 days	2.72 (1.72, 4.31)	4.01 (2.18, 7.40)	0.22	2.38 (1.80, 3.15)	6.03 (3.05, 11.92)	0.03

[a] p value comparing the 3 μg with 3 μg with AlPO$_4$ group.
[b] p value comparing the 6 μg with 6 μg with AlPO$_4$ group.
[c] One case was excluded since outliner data in 6 μg group on Day 42.

eggs.[13] There are currently a number of licensed pandemic and seasonal influenza vaccines, produced in Vero and MDCK cell lines.[14–16] There have also been Phase 1/2 clinical trials of two Vero cell line-derived, and one MDCK cell line-derived H5N1 vaccines.[14,17,18] The safety profile of our candidate vaccine is comparable with the other cell line-derived H5N1 vaccines. The most frequently local side effect reported by our study population was pain (20% to 60%), which was comparable with data from other cell line-derived H5N1 vaccines.[14,17,18] The individual who had mild to moderate elevation of bilirubin level after the first dose of vaccination, did not receive the second dose and had stable bilirubin level after follow-up on Day 210. There were also no SAEs among our study patients, suggesting that our candidate H5N1 vaccine was well tolerated.

In addition to optimizing the manufacturing method, another way to reduce the demand of target antigen is to optimize the immune response. Although adequate immune responses to unadjuvanted split H5N1 vaccines required formulations containing up to 90 μg HA, the use of classical aluminum-based adjuvants was shown to decrease this to 30 μg to 45 μg/dose.[4,17–20] Our data were comparable with these studies and showed that aluminum-based adjuvants played a significant role in enhancing the immune response in the 6 μg group on Day 42. The trend was also noted in the HAI GMT in the 3 μg group as well as in the 6 μg group. Interestingly, these data differed from those from other cell line-derived H5N1 vaccines, where addition of the aluminum adjuvant did not improve, or decreased, the immune response at an antigen dose of 7.5 μg to 15 μg.[14,17] The conflicting effects of aluminum adjuvant on H5N1 vaccines may be explained by the fact that, in addition to the antigen concentration, the immune enhancing effect of the adjuvant may also be dictated by the adjuvant to antigen ratio.[21] MF59 has also been used as an adjuvant and the 3.75 μg H5N1 vaccine containing 50% MF59 was shown to generate a protective immune response, which fulfilled the European criteria for pandemic vaccine licensure.[18] Another oil-in-water emulsion adjuvant formulation as ASO3$_A$ had been used in split-virion H5N1 influenza vaccine and showed protective immune response and also adjuvant effect.[22] Further studies are needed to explore the role of aluminum and other adjuvants in cell line derived-H5N1 vaccines.

We evaluated the neutralization assay as a tool to measure immunogenicity of the vaccine. The HAI assay is not the most efficient method to detect anti-H5 antibodies in avian virus infections,[23–25] and the neutralization assay was previously suggested as a better method to evaluate the immune response in an animal model[26] and in a human study.[27] Our data are consistent with these studies and suggested that the neutralization assay was a more sensitive means of evaluating the immune response. It has been suggested that among criteria to test influenza vaccine during manufacture is to confirm sero-conversion with neutralization titers>1:40 after two shots of different doses of vaccine.[26] Whether such new criteria should be included in further avian influenza vaccine manufacture needs further validation.

In conclusion, our study demonstrated that the H5N1 vaccine produced from the MDCK cell line can be used as a safe alternative to the traditional H5N1 vaccine preparation. Our neutralization assays showed a significant immune response in participants vaccinated with the 6 μg dose with alum adjuvant. It is important to further investigate the possibility of enhancing vaccine immunogenicity with higher antigen doses and to evaluate the role of aluminum or other nonalum adjuvants in vaccine formulations.

References

1. World Health Organization. Cumulative number of confirmed human cases of Avian influenza A/(H5N1) reported to WHO. http://www.who.int/influenza/human_animal_interface/EN_GIP_20120706CumulativeNumberH5N1cases.pdf; [accessed 26.07.12].

2. Keitel WA, Atmar RL. Vaccines for pandemic influenza: summary of recent clinical trials. *Curr Top Microbiol Immunol* 2009;**333**:431–51.

3. Hayden FG, Howard W, Palkonyay L, Kieny MP. Report of the 5th meeting of the evaluation of pandemic influenza prototype vaccines in clinical trials: World Health Organization, Geneva, Switzerland, 12-13 February 2009. *Vaccine* 2009;**27**:4079–89.

4. Leroux-Roels I, Leroux-Roels G. Current status and progress of prepandemic and pandemic influenza vaccine development. *Expert Rev Vaccines* 2009;**8**:401–23.

5. Treanor JJ, Campbell JD, Zangwill KM, Rowe T, Wolff M. Safety and immunogenicity of an inactivated subvirion influenza A (H5N1) vaccine. *New Engl J Med* 2006;**354**:1343–51.

6. Hu AY, Weng TC, Tseng YF, Chen YS, Wu CH, Hsiao S, et al. Microcarrier-based MDCK cell culture system for the production of influenza H5N1 vaccines. *Vaccine* 2008;**26**:5736–40.

7. Tree JA, Richardson C, Fooks AR, Clegg JC, Looby D. Comparison of large-scale mammalian cell culture systems with egg culture for the production of influenza virus A vaccine strains. *Vaccine* 2001;**19**:3444–50.

8. Wright PF. Vaccine preparedness — are we ready for the next influenza pandemic? *N Engl J Med* 2008;**358**:2540–3.

9. Chong P, Chou Frieda, Hsu J, Lee M, Tseng Y, Chiang J, et al. Emergency production of Avian flu vaccines. Poster presentation: Preclinical vaccines and other intervention strategies. International Conference on Options for the Control of Influenza VI. Toronto, Ontario, Canada. June 17-23, 2007. p. 593–595.

10. European Committee for Proprietary Medicinal Products. Note for guidance on harmonisation of requirements for influenza vaccines (CPMP/BWP/214/96). London: European Agency for the Evaluation of Medicinal Products; 1997.

11. Center for Biologics Evaluation and Research. Guidance for industry: clinical data needed to support the licensure of pandemic influenza vaccines. Bethesda: Food and Drug Administration; 2007, May.

12. Stephenson I, Gust I, Pervikov Y, Kieny MP. Development of vaccines against influenza H5. *Lancet Infect Dis* 2006;**6**: 458—60.

13. Initiative for Vaccine Research World Health Organization. Use of cell line for the production of influenza virus vaccines: a appraisal of technical, manufacturing, and regulatory considerations. 2007. http://www.who.int/vaccine_research/ diseases/influenza/WHO_Flu_Cell_Substrate_Version3.pdf; [accessed 29.09.11].

14. Ehrlich HJ, Muller M, Oh HM, Tambyah PA, Joukhadar C, Montomoli E, et al. A clinical trial of a whole-virus H5N1 vaccine derived from cell culture. *N Engl J Med* 2008;**358**: 2573—84.

15. Doroshenko A, Halperin SA. Trivalent MDCK cell culture-derived influenza vaccine Optaflu (Novartis Vaccines). *Expert Rev Vaccines* 2009;**8**:679—88.

16. Szymczakiewicz-Multanowska A, Groth N, Bugarini R, Lattanzi M, Casula D, Hilbert A, et al. Safety and immunogenicity of a novel influenza subunit vaccine produced in mammalian cell culture. *J Infect Dis* 2009;**200**:841—8.

17. Keitel WA, Dekker CL, Mink C, Campbell JD, Edwards KM, Patel SM, et al. Safety and immunogenicity of inactivated, Vero cell culture-derived whole virus influenza A/H5N1 vaccine given alone or with aluminum hydroxide adjuvant in healthy adults. *Vaccine* 2009;**27**:6642—8.

18. Keitel W, Groth N, Lattanzi M, Praus M, Hilbert AK, Borkowski A, et al. Dose ranging of adjuvant and antigen in a cell culture H5N1 influenza vaccine: safety and immunogenicity of a phase 1/2 clinical trial. *Vaccine* 2010;**28**:840—8.

19. Bernstein DI, Edwards KM, Dekker CL, Belshe R, Talbot HKB, Graham IL, et al. Effects of adjuvants on the safety and immunogenicity of an avian influenza H5N1 vaccine in adults. *J Infect Dis* 2008;**197**:667—75.

20. Wu J, Fang HH, Chen JT, Zhou JC, Feng ZJ, Li CG, et al. Immunogenicity, safety, and cross-reactivity of an inactivated, adjuvanted, prototype pandemic influenza (H5N1) vaccine: a phase ii, double-blind, randomized trial. *Clin Infect Dis* 2009; **48**:1087—95.

21. Nicholson KG, Colegate AE, Podda A, Stephenson I, Wood J, Ypma E, et al. Safety and antigenicity of non-adjuvanted and MF59-adjuvanted influenza A/Duck/Singapore/97 (H5N3) vaccine: a randomised trial of two potential vaccines against H5N1 influenza. *Lancet* 2001;**357**:1937—43.

22. Chu DW-S, Hwang SJ, Lim FS, Oh HM, Thongcharoen P, Yang PC, et al. Immunogenicity and tolerability of an AS03A-adjuvanted prepandemic influenza vaccine. A phase III study in a large population of Asian adults. *Vaccine* 2009;**27**:7428—35.

23. Beare AS, Webster RG. Replication of avian influenza virus in humans. *Arch Virol* 1991;**119**:37—42.

24. Hinshaw VS, Webster RG, Easterday BC, Bean WJ. Replication of avian A viruses in mammals. *Infect Immune* 1981;**34**:354—61.

25. Profeta ML, Palladino G. Serological evidence of human infections and avian influenza virus. *Arch Virol* 1986;**90**: 355—60.

26. Zhu H, Ding X, Chen X, Yao P, Xu F, Xie R, et al. Neutralizing antibody but not hemagglutination antibody provides accurate evaluation for protective immune response to H5N1 avian influenza virus in vaccinated rabbits. *Vaccine* 2011;**29**:5421—3.

27. Rowe T, Abernathy RA, Hu-Primmer J, Thompson WW, Lu X, Lim W, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. *J Clin Microbiol* 1999;**37**:937—43.