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Safety and immune responses following administration of H1N1 live attenuated influenza vaccine in Thais

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ABSTRACT

Background: Emergence and rapid spread of influenza H1N1 virus prompted health authorities to develop a safe and effective influenza vaccine for domestic use. The Thai Government Pharmaceutical Organization (GPO) with technical support from Russia through WHO had prepared a pandemic live attenuated vaccine (PLAIV) using *ca-ts* attenuated candidate strain A/17/CA/2009/38 (H1N1) for Thais.
Methods: Each participant received two doses of intranasal H1N1 vaccine or placebo 21 days apart. All were followed up at 7, 21, 42 and 60 days after first immunization. Blood was drawn for hemagglutination inhibition (HAI) assay from all participants at days 1, 21, 42, and 60 after first immunization. A subset of 40 participants aged 19–49 years was randomly selected for nasal washing at days 1, 21, 42, and 60 to assess IgA using direct enzyme-linked immunosorbent assay (ELISA) along with serum HAI and microneutralization (MN) assay determination.
Results: A total of 363 subjects aged 12–75 years were randomized into 2 groups (271 vaccinees:92 placebos). Almost all AEs were mild to moderate. Local reactions were stuffy nose (22.3%), runny nose (25.1%), scratchy throat (27.2%) and sore throat (19.3%). Systemic reactions included headache (21.7%), myalgia (13.8%), fatigue (16.8%) and postnasal drip (19.9%). On day 60, HAI seroconversion rates for vaccine:placebo group were 30.3:6.0 for ITT and 29.4:5.1 for PP analysis. Children showed highest seroconversion rate at 44, but it decreased to 39.4 when all 3 assays (HAI, MN assay and ELISA) from subgroup analysis were considered.
Conclusion: The vaccine candidate is safe. The use of more than one assay may be needed for evaluation of immune response because live attenuated vaccines could effectively induce different kinds of responses. Different individuals could also mount different kinds of immune response, even to the same antigen.

1. Introduction

With the outbreak of H1N1 virus (swine flu) pandemic in Thailand on May 2009, the Thai Government Pharmaceutical Organization (GPO) with technical support from the World Health Organization (WHO) had prepared production of its own pandemic live attenuated vaccine (PLAIV) for Thais. Using *ca-ts* attenuated candidate strain A/17/CA/2009/38 (H1N1), produced by classical genetic reassortment in chicken embryos and prepared at the Institute of Experimental Medicine (IEM) in St. Petersberg, Russia as seed strains, the PLAIV is then produced at GMP-certified pilot plant at the Faculty of Pharmacy, Silpakorn University in Bangkok, Thailand.

Live attenuated influenza vaccines (LAIVs) are considered the "gold standard" for many vaccine-preventable diseases. Administered by nasal spray, LAIVs confer mucosal immunity, creating a first line of defense at the natural point of entry of circulating influenza viruses, and stimulate a potent systemic immune response in the form of both cell-mediated immunity and antibodies [1–4]. There is some evidence from clinical studies that LAIV is more effective than inactivated vaccine in young children [5–7]. During a pandemic, potential protection against drifted strains is

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considered a useful attribute of the vaccine, as it is not known as to what extent the circulating strains of H1N1 might antigenically drift from the vaccine strain over time. LAIV can induce innate immunity through interferon production 1–2 weeks following vaccination thereby protecting children from illnesses associated with circulating respiratory viruses [8]. Along with interferon production, stimulation of innate immunity through natural killer cells may also provide protection particularly when influenza circulates soon after vaccination [9]. For Th1 type responses, antigen-specific production of interferon-gamma (IFN-γ) represents a quantitative marker of protective cell-mediated immune response [10]. In adults, LAIV vaccines induce immune memory that modulates immune response to subsequent heterosubtypic challenge by influencing both innate and adaptive responses [11]. Mass distribution of free intranasal vaccine in community settings such as schools would also be an advantage [12].

The objectives of this study are to evaluate safety, reactogenicity and determine humoral immune response of two doses of intranasal LAIV administered 21 days apart.

2. Materials and methods

2.1. Ethics

Parents of subjects <18 years signed an informed consent form and assent was obtained from every subject <18 years. Subjects aged 18–75 years gave written informed consent. The study was approved by the Ethics Review committees of the Faculty of Tropical Medicine, Mahidol University and the Institute of the Development of Human Research Protections (IHRP), Ministry of Public Health, Thailand.

2.2. Study design and sample size estimation

A randomized double blind, placebo-controlled study was conducted in healthy subjects aged 12–75 years. Eligible subjects were randomly assigned in a 3:1 ratio to H1N1 LAIV candidate strain A/17/CA/2009/38 (H1N1) or placebo by intranasal spray 21 days apart. Estimated sample size was based on the primary endpoint that 70% of vaccinated participants developed seroconversion (4-fold increase in antibody titer compared to baseline, as determined by HAI method). The calculated sample size was 324.

2.3. Study vaccine

The study vaccine is a LAIV strain A/17/CA/2009/38 (H1N1) manufactured by the Thai Government Pharmaceutical Organization (trade name: Flu Vac). The cold adapted, temperature sensitive master donor seed of A/Leningrad/134/57(H2N2) of 21 passages at optimal temperature (32–33 °C) was reasserted with the current pandemic A/California/07/2009(H1N1) to be the pre-master seed of A/17/CA/2009/38. Production and quality control was based on the proposed WHO recommendations for production and control of influenza vaccine (human, live attenuated), 15 June 2009 version.

Vaccine is supplied as a vial containing freeze–dried cake in USP type 1 glass vial. Average thawing time: 10–15 min It is clear to slightly opalescent liquid of colorless to light-yellow color without precipitates and foreign inclusions. Dose is 0.5 mL (0.25 mL per nostril) and administered using a nasal sprayer (LINDAL dispenser, Model ST183, LINDAL Group, Germany). Each vaccine dose contains $10^{6.5–7.5}$ EID_{50} of influenza virus strain A/17/CA/2009/38 (H1N1), lot numbers FV5308, FV5310 and FV5405.

Table 1
Criteria for grading local and systemic adverse events.

Grade	Description
None	No symptoms
Mild	Ill-defined symptoms
Moderate	Symptoms affecting normal daily activity
Severe	Symptoms markedly affecting normal daily activity and needed medication or clinic visit or limited activity

2.4. Placebo

The placebo consists of stabilizers (6.84% sucrose, 1.21% arginine, 1.00% hydrolyzed gelatin, 0.094% glutamate, 1.13% KH_2PO_4, and 0.48% KH_2PO_4) used in vaccine formulation.

2.5. Study subjects

Included were subjects who were anti HIV-negative and seronegative to H1N1 influenza virus with HAI antibody titer ≤ 1:10. Excluded were those with recent influenza infection confirmed as H1N1 with HAI antibody titer > 1:10, hypersensitivity to egg or egg products, medical conditions that predispose to complications from influenza (e.g. lung disease, renal disease, heart disease, metabolic disease such as diabetes), history of asthma, fever, clinically significant illness, known immunosuppressive condition or immune deficiency disease, or any intake of aspirin. All women must have a negative urine pregnancy test at screening and immediately before each vaccination.

2.6. Safety and reactogenicity assessments

2.6.1. Safety assessment

Safety assessment included solicited symptoms, AEs, and antipyretic and analgesic used from the time the consent/assent form was signed through 21 days following first vaccination and with subsequent vaccination through 21 days thereafter. Adverse events were classified as related or not related to the vaccine for both vaccine and placebo groups using MedDRA coding. Diary cards were used to record solicited symptoms, AEs and medications, and were returned back at day 7 after each vaccination.

An adverse event (AE) is defined as (1) any unfavorable and unintended change in the structure, function or chemistry of the body temporally associated with vaccine whether or not considered related to product use and (2) any clinically significant adverse change in frequency and/or intensity of a preexisting condition that is temporally associated with vaccine. Table 1 shows criteria for grading local and systemic adverse events.

A serious adverse event (SAE) refers to any life threatening event that occurs at any dose resulting in (1) death, (2) persistent or significant disability/incapacity (3) or prolongs an existing inpatient hospitalization (hospitalization is defined as an inpatient admission regardless of length of stay, even if hospitalization is a precautionary measure for continued observation), (4) congenital anomaly/birth defect (in offspring of subject taking the product regardless of time of diagnosis) and (5) overdose (accidental or intentional).

2.6.2. Reactogenicity assessment

Vital signs or temperature alone was recorded twice daily for 7 days after each immunization and with each follow up visit. Oral temperature (T) was taken every 4 h in those with fever $T > 37.5$ °C. Grading of temperature was 0 (no) for $T \leq 37$ °C; 1 (mild) for $T > 37$ °C to ≤37.5 °C; 2 (moderately high) for $T > 37.5$ °C to ≤38.5 °C; 3 (high) for $T > 38.5$ °C.

Medication or treatment for either reactogenicity or adverse events was coded using WHO-diagnosis-related group (DRG) system.

2.7. Humoral immune response assays

Blood collection and nasal washing were done at days 1, 21, 42 and 60 to evaluate antibody response against vaccination. Only a subgroup of 40 subjects from age group 19–49 years was randomly selected to do nasal washing. HAI assay was performed using sera of all subjects whereas MN assay was selectively performed using sera of 40 participants whose nasal wash was collected.

2.7.1. HAI assay

HAI assay was performed as previously described [13,14]. Prior to assay, sera was treated with receptor destroying enzyme (RDE) (Denka Seiken, Tokyo, Japan) to eliminate non-specific inhibitors and absorbed with 50% goose erythrocytes to remove non-specific agglutinators. A/17/CA/2009/38 (H1N1) at concentration of 4 HA units/25 μl was used as test virus and 0.5% goose erythrocytes were used as indicator for HAI assay. HAI antibody titer was defined as reciprocal of the highest serum dilution that completely inhibits hemagglutination reaction. Positive control serum and back titration of the test virus were included in every experiment run.

2.7.2. Microneutralization assay

An ELISA-based MN assay was performed on MDCK cell monolayer as described previously [13,14]. Sera was treated with RDE in the same manner as for HAI assay but absorption with goose erythrocytes was omitted. A/CA/sera 07/2009 (H1N1) or A/Thailand/104/2009 (H1N1)) at final concentration of 100 TCID50/100 μl was used as test viruses. Presence of influenza A nucleoprotein in infected cell monolayer was assayed by ELISA using mouse specific monoclonal antibody (Chemicon, Temecula, CA) as primary antibody and goat anti-mouse Ig (Southern Biotech, Birmingham, AL) as secondary antibody. Antibody titer is defined as the reciprocal of the highest serum dilution that reduces ≥50% of the amount of viral nucleoprotein in reaction wells compared to virus control wells.

2.8. Detection of IgA

Indirect ELISA was used to detect IgA in nasal wash. Each well of microtiter plate (96-well Maxisorp plates, Nunc^{TM}, Denmark) was coated with 100 μl of influenza HA antigen (A/California/7/2009 (H1N1) v (NYMCX-179A), Cell Derived, NIBSC code 09/174, UK) at concentration of 2 μg HA/ml in PBS and incubated overnight at 4 °C. After 3 washes with 0.05% Tween-20 in PBS (PBS-Tween), coated plate was blocked with 200 μl of PBS-Tween containing 4% fetal bovine serum (FBS) at room temperature for 1 h. After 3 washes, 100 μl of nasal wash diluted in PBS-Tween containing 1% FBS was added to the plate starting at dilution of 1:10, followed by incubation at 37 °C for 2 h. After washings, 100 μl of peroxidase-conjugated goat anti-human IgA (Sigma–Aldrich Chemicals, St Louis, MO) (50,000-fold diluted in PBS-Tween containing 1% FBS) was added. After incubation at 37 °C for 1 h, the plate was washed six times, and 100 μl of *O*-phenylenediamine dihydrochloride (Sigma–Aldrich Chemicals, St Louis, MO) was added to reaction wells and incubated at room temperature for 10 min for color development. One hundred microliters of 1 N sulphuric acid solution was added to stop the reaction. Optical density at 490 nm (OD_{490}) was determined by a microtiter plate reader. A positive result was considered when fold difference in OD values between paired nasal washes collected before and after vaccination was equal or 2× greater.

2.9. Statistical methods for data analysis

2.9.1. Parametric and non-parametric statistics

Analysis was based on unpaired comparisons between two or three groups. Chi-square test, Kruskal Wallis test, Mann Whitney *U*-test and one-way ANOVA (with post hoc analysis by Bonferroni correction) were applied. Level of statistical significance was set at p-value < 0.05. SAS version 9.2 was used for analysis.

2.9.2. Data analyses considerations

a. Population for analysis

All subjects who received at least one vaccination were included in the safety population. This population was used in evaluating safety and tolerability.

b. Analysis of data sets

Efficacy analysis was conducted based on an intention-to-treat (ITT) analysis. In some individuals who did not comply with the protocol-defined treatment schedule, a per-protocol analysis (PP) was used.

Definition of immunological end point: vaccine is able to induce 4-fold rise of HAI in 40–70% of vaccinees 60 days after the first immunization.

3. Results

3.1. Study subjects

A total of 1048 potential subjects were screened, of which 363 were enrolled and randomized into 2 groups using a 3:1 ratio (271 vaccinees:92 placebos). Subjects were stratified into three age groups: 12–18 years (110 subjects), 19–49 years (120 subjects) and 50–75 years (133 subjects). However, only 244 who received the vaccines were included in the ITT analysis: 75 (12–18 years), 86 (19–49 years) and 83 (50–75 years) (Fig. 1). In the placebo group, 83 were included in the ITT analysis: 25 (12–18 years), 30 (19–49 years) and 28 (50–75 years). Per-protocol (PP) population ratio for vaccine:placebo was 228:78 and was stratified into three age groups as 66:22 (12–18 years), 85:29 (19–49 years) and 77:27 (50–75 years).

Significantly, more females (56.1%, 68.5%, 91%) were in the vaccine group of all age groups (12–18 years, 19–49 years, 50–75 years, respectively). In the placebo group, more females were in all age groups except 19–49 years. There was no significant difference among the stratified age groups.

3.2. Safety profile

The study vaccine is safe. Almost all reported AEs were mild to moderate. Three cases with severe AEs in vaccine group had sleepiness, cough and abdominal pain. All events were considered not serious. There was no reported SAE. At least one AE was experienced by 185 of 271 participants in the vaccine group and 64 of 92 participants in the placebo group. There were a total of 391 and 138 events in vaccine and placebo groups, respectively. Among 391 events in vaccine group, 195 (49.9%) were reported as probably related or probably not related.

The younger age group (12–18 years) had more upper respiratory tract adverse reactions (57.9% vaccine group, 65.4% placebo group) compared with 19–49 (36.0%, 35.9%) and 50–75 (34%, 39.0%). However, this difference was not statistically significant ($p = 0.3694$).

In all age groups, most reported reactions decreased after second immunization (Fig. 2). Scratchy throat (29.5%, 20.5%, $p = 0.1$), stuffy and runny nose (21.7%, 24.1%, $p = 0.65$), and sore throat

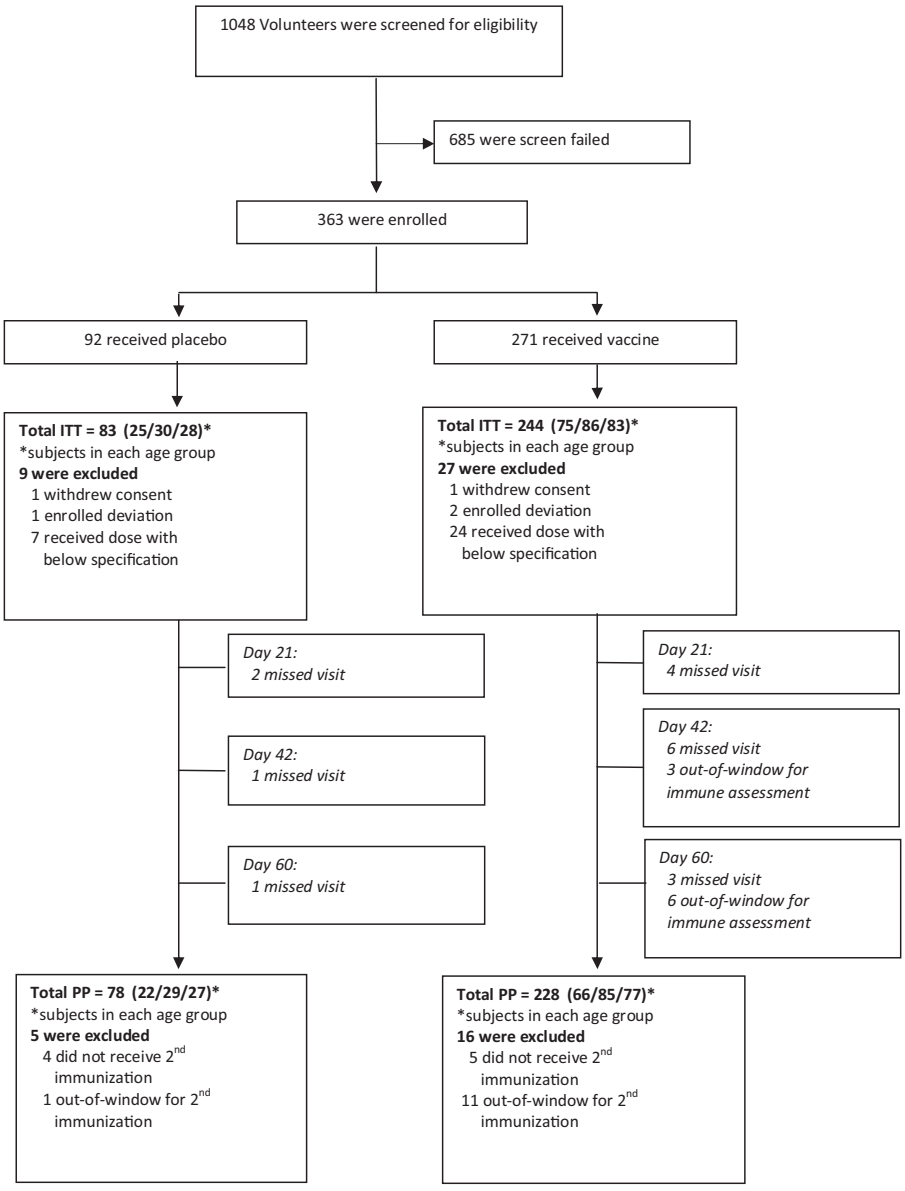

Fig. 1. Subject disposition for the study.

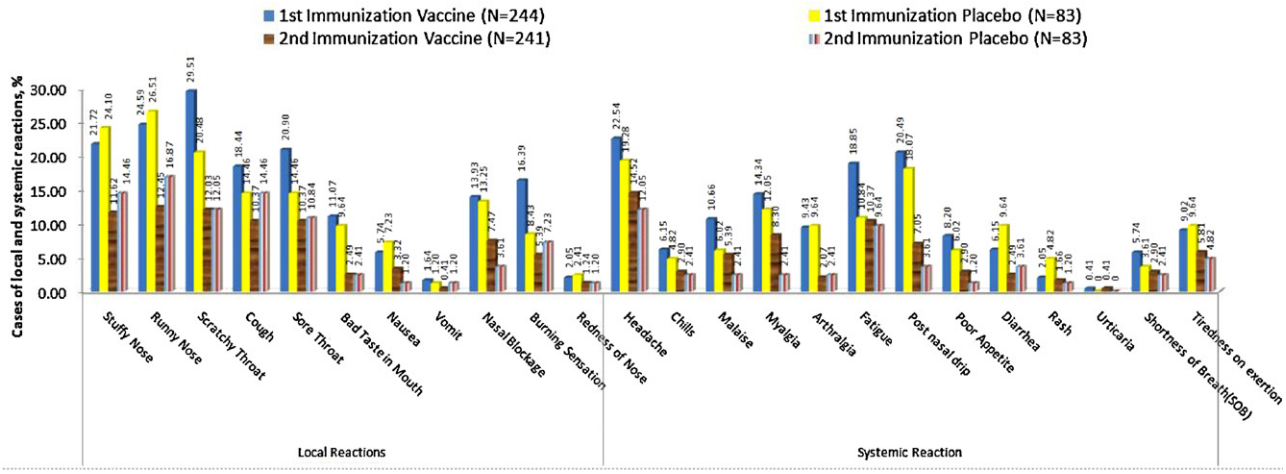

Fig. 2. Local and systemic reactions in vaccine and placebo groups during first and second immunization.

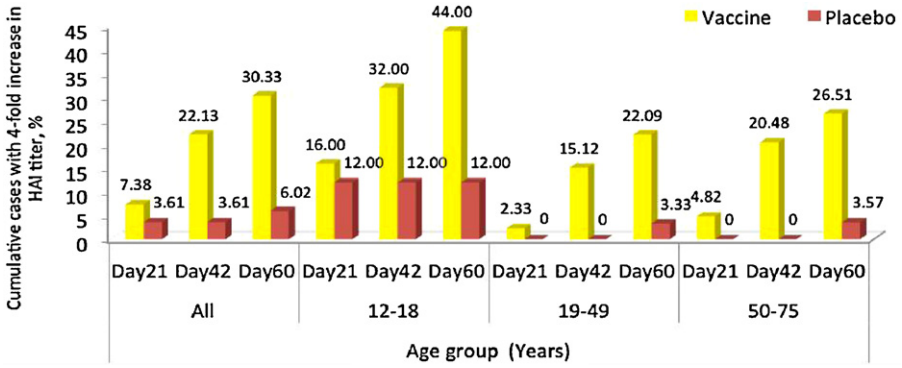

Fig. 3. Immune response: intention-to-treat analysis in study participants with 4-fold increase in titer.

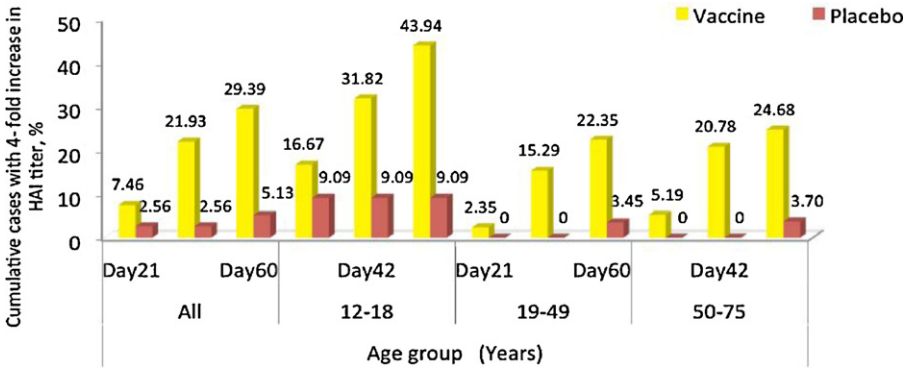

Fig. 4. Per-protocol analysis in study participants with 4-fold increase in titer.

(20.9%, 14.5%, $p = 0.2$) were the most frequently reported local reactions in the vaccine and placebo groups, respectively in the first immunization. Headache (22.5%, 19.3%, $p = 0.533$), myalgia (14.3%, 12.5%, $p = 0.6$), fatigue and postnasal drip were the most frequently reported systemic reactions in the vaccine and placebo groups, respectively in the first immunization. Most cases had no fever on vaccination day (day 1, day 21).

3.3. Humoral immune response

Analysis of HAI test was performed for both ITT and PP populations. The ITT analysis included all cases of ITT population with immune responses regardless of whether assessment (at days 21, 42, 60) was out-of-window or not. PP analysis showed that immune response assessment at days 21, 42, 60 was within their respective window periods (Figs. 3 and 4).

A subset of 40 participants (29 vaccinees and 11 placebo) from age group 19–49 was randomly chosen for antibody detection using MN assays (in serum) and ELISA (in nasal wash) in addition to HAI assay.

When all 3 assays (HAI, MN, ELISA) were considered, antibody positive rates were 7.1%, 25.0% and 39.3% from samples taken at days 21, 42, and 60, respectively (Table 2).

4. Discussion

This study showed that the vaccine is safe. This finding was consistent with other studies [8,15–26]. Local reactions include stuffy and runny nose, scratchy and sore throat (21.7%, 29.5% and 20.9%, respectively) and common systemic reactions include headache, myalgia and fatigue in vaccinees. Similar reactions were reported in studies in the US and India [17,27,28]. Local and systemic reactions on vaccination day or 7 days post-vaccination appeared not to

Table 2
Results from 3 assays (HAI, MN, ELISA) with ≥4-fold increase in titer in a subset of 40 participants.[a]

Day	*n*	Status	Positive with 1 test alone[b]			Positive with more than 1 test[b]				Total
			HAI	MN	ELISA	HAI/MN	HAI/ELISA	MN/ELISA	HAI/MN/ELISA	
21	28[c,e]	Vaccinees	–	–	A[1]	–	–	B[1]	–	2 (7.1%)
	11	Placebos			a[1]					1 (9.1%)
42	28[c,e]	Vaccinees	C[1]	D[1]	AE[2]	BFG	–	–	–	7 (25%)
	10[d,e]	Placebos		b[1]						1 (10%)
60	28[c,e]	Vaccinees	CH[2]	I[1]	AJK[3]	BDFGL[5]	–	–	–	11 (39.3%)
	11	Placebos				b[1]				1 (9.1%)

[a] 29 vaccinees, 11 placebos.
[b] Each letter corresponds to a study participant.
[c] Missed visit by 1 vaccinee on day 21, day 42, and day 60.
[d] Missed visit by 1 placebo on day 42.
[e] All vaccinees and placebos with missed visits did not show immuno-reactivity against the vaccine.

be different between the 2 groups. Most reactions decreased after second immunization, which was consistent with another study [29]. While 1.5% of H1N1 LAIV recipients had fever ($T \geq 38.3\,^{\circ}C$) after the first dose of vaccine in this study [29], most cases (vaccine and placebo) in our study had no fever ($T \geq 38\,^{\circ}C$) on vaccination day (day 1, day 21).

A higher immune response rate in the younger (12–18) age group compared to older age group was demonstrated in our study. Children showed higher rates of HAI seroconversion (44.59%) at day 60. These findings were similar with results from clinical trials conducted by Rudenko LG using similar product (personal communication). Studies in children have shown that LAIV provides greater protection compared to inactivated influenza vaccines. A study in children aged 6–36 months showed HAI seroconversion in 61% of LAIV recipients compared to 13% placebo ($p < 0.001$) [9]. In our study, presence of pre-existing immunity not detected during enrollment could not be responsible for the differences observed because only subjects who were seronegative to H1N1 influenza virus (HAI titer $\leq$ 1:10) were included in the study. While antibodies present in the serum and mucosal surfaces are good correlates of immunity to influenza for children and young adults, cytokine production and proliferation of T cells particularly CD4+ cells maybe more important in the older age group [30]. Furthermore, in the elderly, cytotoxic T-cell responses associated with granzyme B production correlate better with protection than antibody [31,32]. Previous observations in children aged 5–9 years given a single intranasal dose of LAIV elicited significant mean increases in percentage of influenza A virus-reactive IFN-γ positive cells in T-lymphocyte and NK cell subsets, as measured by flow cytometry [33]. Therefore, LAIV may elicit a long-lasting, broader immune (humoral and cellular) response similar to natural immunity [34].

Children are highly susceptible to influenza virus infection and clinical disease. School-aged children experience high rates of influenza infection, febrile illness, and school absenteeism [35]. In addition, there is greater economic burden on families with influenza-like illnesses as medical expenses are higher along with missed schooldays and lost workdays for parents [36]. For children, intranasal LAIV remains more acceptable than parenteral vaccine from a practical and psychological point of view [23].

Our study showed that HAI titer was lowest when all 3 assays were compared. Experimental and epidemiologic data showed that protective properties of LAIVs correlate poorly to antibody titers determined by traditional HAI assay. It has been shown that intranasal vaccine-induced IgA antibody response in respiratory secretions and mucosal IgA antibodies correspond with protection against influenza virus infection. Aside from IgA ELISA, other methods for evaluation include IgG ELISA and cytokine assays consistent with the recently updated WHO recommendations on LAIV monitoring [37].

The immune response study was expanded in 40 randomly selected participants of age group 19–49 years. We detected IgA in nasal secretions that might be locally- or serum-derived. When only HAI result was considered, a positive rate of 30 was noted at day 60, but when HAI, MN and ELISA results were analyzed together, the rate increased to 39.3. This suggests that there are other types of antibodies stimulated by LAIV. Mucosal (nasal) IgA antibody responses and a strong cell-mediated immune response could have been elicited. The MN assay utilized A/CA/07/2009 (H1N1) and A/Thailand/104/2009 (H1N1), which are wild type viruses as test antigens. A positive response in antibody responses was observed in vaccinees with 4-fold increase in antibody titer compared to baseline. This indicated that LAIV vaccine had induced immune response that cross-reacts with wild type circulating strains.

This vaccine was licensed for pandemic use. This work supported WHO's mission of strengthening pandemic influenza preparedness in developing countries through technology transfer in the production of influenza vaccine.

5. Conclusion

The vaccine candidate is safe. Difference in response rates between vaccine and placebo groups of age group 12–18 years was higher than other groups. The use of more than one assay may be needed for evaluation of immune response after vaccination as LAIVs could effectively induce mucosal antibody responses and T-cell responses that may provide protective immunity. Different individuals could also mount different kinds of immune response, even to the same antigen.

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