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# Preliminary Study about Sublingual Administration of Bacteria-expressed Pandemic H1N1 Influenza Vaccine in Miniature Pigs

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**Sublingual (SL) administration of influenza vaccine would be non-invasive and effective way to give human populations protective immunity against the virus, especially when pandemic influenza outbreaks. In this study, the efficacy of pandemic influenza virus-based subunit vaccines was tested after sublingual (SL) adjuvant administration in pigs. Eight specific pathogen-free Yucatan pigs were divided into 4 groups: non-vaccinated but challenged (A) and vaccinated and challenged (B, C, and D). The vaccinated groups were subdivided by vaccine type and inoculation route: SL subunit vaccine (hemagglutinin antigen 1 [HA1] + wild-type cholera toxin [wtCT], B); IM subunit vaccine (HA1 + aluminum hydroxide, C); and IM inactivated vaccine (+ aluminum hydroxide, D). The vaccines were administered twice at a 2-week interval. All pigs were challenged with pandemic influenza virus (A/swine/GCVP-KS01/2009 [H1N1]) and monitored for clinical signs, serology, viral shedding, and histopathology. After vaccination, hemagglutination inhibition titre was higher in group D (320) than in the other vaccinated groups (40–80) at the time of challenge. The mobility and feed intake were reduced in group C. Both viral shedding and histopathological lesions were reduced in groups B and D. Although this study has limitation due to the limited number of pigs (2 pigs per a group), the preliminary data in this study provided the protective potential of SL administration of bacteria-expressed pandemic H1N1 influenza vaccine in pigs. There should be additional animal studies about effective adjuvant system and vaccine types for the use of SL influenza vaccination.**

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## Introduction

Recently, a new influenza A (H1N1) virus emerged among humans in California and Mexico. Propagation of the virus resulted in pandemic influenza infection within few months from first outbreak through human-to-human transmission, mainly international air travel (Center for disease control and prevention, 2009; Garten *et al*., 2009; Scalera and Mossad, 2009; Smith *et al*., 2009). Around 60% of the patients presenting with H1N1 influenza virus infection were younger than 18 years (Novel Swine-Origin Influenza A (H1N1) Virus Investigation Team, 2009), which reinforces the importance of population-based control of pandemic influenza virus.

The new H1N1 virus is similar to North American swine H1N1 viruses rather than to seasonal human influenza H1N1 viruses (Garten *et al*., 2009). Notwithstanding the close relation, evidence of pig-to-human transmission is yet to be found (Vallat, 2009). Several reports state that pandemic H1N1 influenza viruses were found in swine farms through human-to-pig transmission (Moreno *et al*., 2010; Song *et al*., 2010; Sreta *et al*., 2010).

Vaccination is an effective strategy to protect humans and pigs from pandemic influenza viruses. Ferrets immunized with 2 doses of 2009 A (H1N1) inactivated vaccines were protected from infections of pandemic H1N1, but not seasonal H1N1 influenza viruses (Shin *et al*., 2010). However, classical swine influenza virus conferred cross-protection for pandemic influenza viruses in mice and ferrets (Min *et al*., 2010). Multiple infection by seasonal influenza A viruses or inactivated trivalent seasonal influenza vaccination were also reported to induce some cross-protection for pandemic influenza virus in ferrets (Laurie *et al*., 2010; Lee *et al*., 2010). Recently, bacteria-expressed hemagglutinin antigen 1 (HA1)-based protein or HA1 fused with flagellin [a Toll-like receptor 5 (TLR5) ligand] were studied for their potential as subunit vaccines (Aguilar-Yanez *et al*., 2005; Song *et al*., 2008a). However, bacterial expression systems lack the machinery for glycosylation, and glycosylation relates to the immunogenicity of recombinant viral proteins, it was reported that bacteria-expressed HA1-based recombinant proteins could also elicit good protective immune responses against pandemic influenza virus challenges in mice and ferrets (Aguilar-Yanez *et al*., 2005; Song *et al*., 2008a).

Most vaccines for influenza virus have been administered via the intramuscular (IM) route. However, several studies have considered the use of other administration routes to

reduce invasiveness of vaccination. The intranasal route is efficient to induce strong cellular and humoral immune responses, but carries the risk of retrograde transport of the adjuvant to the central nervous system (Armstrong *et al*., 2005). As an alternative, sublingual (SL) administration of influenza virus vaccines has been explored and has been shown to be an effective route for mucosal and systemic immune responses in mice (Song *et al*., 2008b). Compared to small animals, large animals (such as the pig) can be helpful in the prediction of the efficacy of SL application in humans.

Classical swine influenza virus vaccines may induce cross-neutralizing antibodies for pandemic influenza virus (Dürrwald *et al*., 2010; Vincent *et al*., 2010), but pandemic influenza virus-based vaccine is preferred for its homology. In this study, pandemic influenza virus-based subunit vaccines were prepared for pigs, and their efficacy after IM or SL administration was compared.

## Materials and Methods

### Virus, animal, and ethical statement

The virus used for preparation of inactivated vaccine and challenge was the A/swine/GCVP-KS01/2009 (H1N1) virus isolated from pigs in a commercial swine farm (KY farm) in Korea. According to the permission from the farm, the virus isolation had been done from nasal swab samples from pigs which had been submitted to Research unit of Green Cross Veterinary Product in Korea as a diagnostic purpose. The sequence similarities between A/swine/GCVP-KS01/2009 (H1N1) and A/California/04/2009 in the hemagglutinin (*HA*) and neuraminidase (*NA*) genes were 99% and 99.3%, respectively. Eight specific pathogen-free (SPF) Yucatan miniature pigs (Medipig, Korea) were employed in this study. The SPF pigs in the study comprised 4 groups of pigs housed in different rooms of the isolation facility at Green Cross Veterinary Products (Korea). All animal experiments complied with the current laws of South Korea. Animal care and treatment were conducted in accordance with guidelines established by the Green Cross Veterinary Products Institutional Animal Care and Use Committee (GCVP-IACUC). This study was approved by GCVP-IACUC with a permit number, GCV-10-4-02.

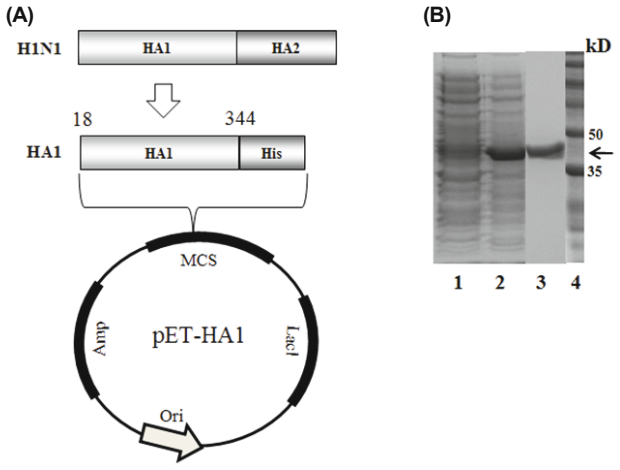


**Fig. 1. Construction, expression, and purification of the HA1 protein.** (A) Schematic for the construction of the HA1 protein. The consensus sequence of HA1 of the influenza A/H1N1/2009 virus was cloned into the pET-21d vector. (B) Expression and purification of the HA1 protein. The protein was analysed by SDS-PAGE. Lanes: 1, before IPTG induction; 2, after IPTG induction; 3, purified protein; and 4, protein molecular weight markers. The arrow indicates the position of the HA1 protein in the SDS-PAGE gel.

### Cloning, expression, and purification of the HA1 protein

The cDNA of HA of the influenza A/California/04/2009 (H1N1) virus was synthesized by GenScript Co., Piscataway, NJ 08854, USA. HA1 (amino acids 18–344) was amplified by PCR using a forward primer (5′-GAATTCCCGACACA TTATGTATAG-3′) and a reverse primer (5′-CTCGAGTC TAGATTGAATAGACGG-3′) to introduce *Eco*RI and *Xho*I restriction enzyme sites. The PCR product digested with *Eco*RI and *Xho*I was ligated into the pET-21d (+) vector (Novagen, USA), resulting in the plasmid pET-HA1.

Recombinant protein was expressed in the *Escherichia coli* BL21 (DE3) strain after transformation with the pET-HA1 plasmid (Fig. 1). The cells were grown in 1 L of LB medium containing 100 mg/ml of ampicillin at 37°C until the optical density at 600 nm ($OD_{600}$) reached 0.6. Isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to a final concentration of 0.5 mM. Cells were incubated for an additional 3 h at 37°C and finally harvested by centrifugation at 6,000 rpm for 15 min. The cell pellet was suspended in lysis buffer (20 mM Tris-HCl; pH 8.0, 50 mM NaCl, 1 mM $MgCl_2$, and 0.1 mM $CaCl_2$). After sonication, the soluble and insoluble fractions were separated by centrifugation at 20,000 rpm for 30 min. The insoluble fraction was dissolved in binding buffer (20 mM Tris-HCl; pH 7.9, 0.5 M NaCl, and 6 M urea). After centrifugation at 20,000 rpm for 30 min, the supernatant was applied to a Talon metal affinity column (Clontech, USA). The column was washed with binding buffer containing 20 mM imidazole, and protein was eluted with elution buffer (20 mM Tris-HCl; pH 7.9, 0.5 M NaCl, and 0.5 M imidazole). The eluate was dialyzed against 1× PBS. Protein concentration was determined by using the Bio-Rad protein assay, and protein aliquots were stored at -80°C.

### Experimental vaccine for pandemic influenza virus

The inactivated vaccine was a monovalent aqueous vaccine containing a total of 5.5 log $TCID_{50}$/ml of formalin-inactivated A/swine/GCVP-KS01/2009 (H1N1) from Madin-Darby canine kidney (MDCK) cells, and aluminum hydroxide-based Rehydragel® (General Chemical, Co., USA) as an adjuvant. The adjuvant was mixed with the inactivated virus to a final concentration of 10% according to the manufacturer's recommendation.

The subunit vaccine was prepared from cloned and purified HA1 proteins that originated from A/California/04/2009 (H1N1). For SL administration, the HA1 protein was mixed with wild-type cholera toxin (wtCT) (List Biological Laboratories, Inc., USA) as adjuvant. For IM administration, the HA1 protein was mixed with the aluminum hydroxide-based Rehydragel® (General Chemical, Co.) as adjuvant.

### Experimental design

Eight SPF-free Yucatan pigs were divided into 4 groups: non-vaccinated but challenged (A) and vaccinated and challenged (B, C, and D). The vaccinated groups were subdivided by vaccine type and inoculation route: SL subunit vaccine (HA1 + wtCT, B); IM subunit vaccine (HA1 + aluminum hydroxide, C); and IM inactivated vaccine (+ aluminum hydroxide, D).

The vaccines were administered twice at a 2-week interval. Two weeks after the second administration, 3 ml of the pandemic influenza virus (5.5 log $TCID_{50}$/ml) was administered intranasally to all pigs. After the challenge, feed intake, mobility, and body temperature was monitored daily for 7 days. The feed intake and mobility were monitored twice a day and scored from 1 to 5 (1, very poor; 2, poor; 3, moderate; 4, good; 5, very good). The body temperature was estimated twice a day by a rectal probe.

To evaluate humoral immune responses, blood was withdrawn at the time of each vaccination, challenge, and every day for 7 days after the challenge. Nasal swabs were also collected every day after the challenge to evaluate viral shedding.

All pigs were euthanized at day 7 post challenge and subjected to necropsy for gross examination of lesions. The lungs and trachea were fixed with 10% formalin for histopathological analyses. The lung scores were calculated according to different scoring systems (Table 4).

### Hemagglutination inhibition (HI) test

Pig sera were tested for HI, i.e. the ability of the sera to inhibit the hemagglutinating activity of the influenza virus. The HI test was performed according to procedures recommended by the World Organization for Animal Health (OIE, 2010). Briefly, 25 µl of serial two-fold dilutions of the treated serum samples were mixed with the same volume of 4 HA units of virus in V-shaped microtitre plates and incubated at room temperature for 30 min. Then, 50 µl of 0.5% chicken red blood cells were added to each well and incubated at room temperature for 40 min. The HI titre was expressed as the reciprocal of highest serum dilution that completely inhibited hemagglutination of 4 HA units of the virus.

### Real-time reverse transcriptase (RT)–PCR for viral titre in nasal swabs

Nasal swab samples were suspended in 1 ml of sterilized phosphate-buffered saline (PBS). From the suspended sample, 250 µl was used for RNA extraction and cDNA synthesis. The RNA and cDNA were prepared using TRIzol® LS (Invitrogen, USA) and M-MLV (Invitrogen) according to the manufacturer's recommendations. To detect the swine influenza virus, primers for the matrix (*M*) gene were used. The Primers and protocols for real-time RT-PCR were the same as those in a previous study (Suwannakarn *et al*., 2008).

**Table 1. Mobility, feed intake, and body temperature in different vaccination groups after challenge of pandemic influenza virus isolate (A/swine/GCVP-KS01/2009 (H1N1))**

| Observations | Group[a] | Individual pigs | Days post challenge | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| Mobility[b] | A | 1 | 4.0 | 3.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| | | 2 | 4.0 | 3.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| | B | 3 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.0 |
| | | 4 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.0 | 3.0 |
| | C | 5 | 4.0 | 4.0 | 4.0 | 3.0 | 3.0 | 2.5 | 3.0 | 3.0 |
| | | 6 | 4.0 | 4.0 | 4.0 | 3.0 | 3.0 | 2.5 | 3.0 | 3.0 |
| | D | 7 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| | | 8 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| Feed intake[b] | A | 1 | 4.0 | 3.0 | 3.5 | 3.5 | 4.0 | 4.0 | 4.0 | 4.0 |
| | | 2 | 4.0 | 3.0 | 3.5 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 |
| | B | 3 | 2.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.0 |
| | | 4 | 2.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.0 |
| | C | 5 | 2.0 | 4.0 | 3.5 | 2.0 | 1.5 | 2.5 | 3.0 | 2.0 |
| | | 6 | 2.0 | 4.0 | 2.0 | 1.0 | 1.5 | 2.5 | 3.0 | 2.0 |
| | D | 7 | 3.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.5 | 4.0 | 4.0 |
| | | 8 | 3.0 | 4.0 | 4.0 | 4.0 | 4.0 | 3.5 | 4.0 | 4.0 |
| Body temperature (°C) | A | 1 | 37.7 | 39.6 | 39.7 | 39.1 | 39.0 | 38.6 | 38.6 | 38.7 |
| | | 2 | 38.1 | 38.3 | 38.2 | 38.1 | 38.1 | 37.6 | 37.7 | 38.1 |
| | B | 3 | 37.3 | 37.8 | 37.7 | 37.8 | 38.0 | 38.1 | 37.7 | 37.8 |
| | | 4 | 37.7 | 37.9 | 37.9 | 37.9 | 38.1 | 38.4 | 38.3 | 38.1 |
| | C | 5 | 37.8 | 38.6 | 38.5 | 38.6 | 38.6 | 38.6 | 38.5 | 38.2 |
| | | 6 | 36.9 | 36.8 | 37.1 | 38.7 | 38.0 | 37.4 | 37.4 | 37.1 |
| | D | 7 | 37.7 | 37.9 | 37.9 | 38.0 | 38.1 | 37.5 | 37.8 | 37.7 |
| | | 8 | 37.6 | 38.0 | 37.8 | 37.7 | 37.7 | 37.4 | 37.5 | 37.5 |

[a] Group A, non-vaccinated and challenged group; group B, SL subunit vaccine (HA1 + wtCT); group C, IM subunit vaccine (HA1 + aluminum hydroxide); and group D, IM inactivated vaccine (+ aluminum hydroxide).
[b] 1, very poor; 2, poor; 3, moderate; 4, good; 5, very good

**Table 2.** HI titres for pandemic influenza virus

| Group[a] | Individual pigs | HI titres | | | | |
|---|---|---|---|---|---|---|
| | | 1st Vaccination | 2nd Vaccination | Challenge | 3 days post challenge | 7 days post challenge |
| A | 1 | <20 | <20 | <20 | <20 | 640 |
| | 2 | <20 | <20 | <20 | <20 | 320 |
| B | 3 | <20 | 40 | 80 | 40 | 80 |
| | 4 | <20 | 40 | 80 | 40 | 320 |
| C | 5 | <20 | 40 | 80 | 80 | 160 |
| | 6 | <20 | 40 | 40 | 80 | 320 |
| D | 7 | <20 | 80 | 320 | 320 | 320 |
| | 8 | <20 | 80 | 320 | 320 | 640 |

[a] Group A, non-vaccinated and challenged group; group B, SL subunit vaccine (HA1 + wtCT); group C, IM subunit vaccine (HA1 + aluminum hydroxide); and group D, IM inactivated vaccine (+ aluminum hydroxide).

The $C_t$ value (obtained by real-time RT-PCR) was converted into 50% egg infective dose ($EID_{50}$) based on values of the standard control (serially diluted swine influenza A/H1N1 virus).

## Results

### Comparison of clinical parameters after pandemic influenza virus (H1N1) challenge

After experimental challenge with the pandemic influenza virus (H1N1), the mobility, feed intake, and body temperature were monitored in group A (non-vaccinated and challenged group), group B (subunit vaccine [HA1 + wtCT] via SL route), group C (subunit vaccine [HA1 + aluminum hydroxide] via IM route), and group D (inactivated vaccine (+ aluminum hydroxide) via IM route) (Table 1). The relatively poor scores (1–2) in feed intake were observed in group C (subunit vaccine + aluminum hydroxide via IM route) after challenge compared with the other groups. The body temperature over 39°C was not observed after challenge in groups B, C, and D. On the other hand, in one pig of group A, the body temperature was increased more than 39°C from 1 to 3 days post challenge.

### Comparison of HI titres

At the time of the challenge (2 weeks after the second vaccination), the mean HI titre in group D (320 titre of HI units) was higher than that in the other groups (Table 2). Although positive HI titres in group B and C were found at this time, these were always below 100. The non-vaccinated group A remained seronegative for influenza virus until the time of challenge. One week after the challenge, influenza virus-specific HI titres were increased in all groups. While HI titres were in the range of 80–320 in group B and C, group A and D exhibited HI titres in the range of 320–640.

### Nasal shedding of challenged influenza virus

After the challenge, nasal shedding of the challenged virus was monitored in groups A–D (Table 3). The duration of viral shedding was 4–6 days in groups A, B, and C, but 2–4 days in group D. The non-vaccinated control group shed viruses 1 day after the challenge, with a peak at 3.2–3.9 $\log_{10}$ $EID_{50}$/ml. The viral shedding was stopped at day 7 post challenge. With <0.5 to 3.0 $\log_{10}$ $EID_{50}$/ml of viral titre, the viral shedding in group B was lower than that in the control group during the shedding time. Although viral shedding was reduced during the last 3 days of the challenge study, no obvious reduction was observed in group C. Group D began to shed viruses with viral titres between 1.5 and 2.1 $\log_{10}$ $EID_{50}$/ml at 3 days post challenge, but viral shedding was gradually decreased until 7 days post the challenge, showing viral titres between <0.5 and 1.8 $\log_{10}$ $EID_{50}$/ml.

### Pathological findings

The lungs were grossly examined for lesions, and histopathological studies were also performed. The lung scores

**Table 3.** Viral shedding after the challenge in the different vaccination groups

| Group[a] | Individual pigs | $LogEID_{50}$/ml in each day post challenge | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | 0 | 1 | 2 | 3 | 4 | 5 | 6 | 7 |
| A | 1 | <0.5 | 3.0 | 3.7 | 3.7 | 3.9 | 2.5 | 1.5 | <0.5 |
| | 2 | <0.5 | 3.0 | 2.2 | 3.2 | 2.4 | 2.8 | 2.0 | <0.5 |
| B | 3 | <0.5 | <0.5 | 2.6 | <0.5 | 1.6 | 1.8 | <0.5 | <0.5 |
| | 4 | <0.5 | 3.0 | 1.1 | 1.7 | 2.4 | 2.6 | 0.9 | <0.5 |
| C | 5 | <0.5 | 2.2 | 3.1 | 2.9 | 2.8 | 1.2 | 1.0 | <0.5 |
| | 6 | <0.5 | 3.2 | 2.1 | 2.4 | 3.5 | 1.1 | 0.9 | <0.5 |
| D | 7 | <0.5 | <0.5 | <0.5 | 2.1 | 1.8 | 1.5 | 1.0 | <0.5 |
| | 8 | <0.5 | <0.5 | <0.5 | 1.5 | 1.8 | <0.5 | <0.5 | <0.5 |

[a] Group A, non-vaccinated and challenged group; group B, SL subunit vaccine (HA1 + wtCT); group C, IM subunit vaccine (HA1 + aluminum hydroxide); and group D, IM inactivated vaccine (+ aluminum hydroxide).

**Table 4. Lung scores at 1 week after challenge with the pandemic influenza virus isolate (A/swine/GCVP-KS01/2009 (H1N1))**

| Group[a] | Individual pigs | Histopathological findings and scores[b] | | | Total score |
|---|---|---|---|---|---|
| | | Interstitial pneumonia | Bronchial pneumonia | Suppurative tracheitis | |
| A | 1 | 3 | 2 | 1 | 6 |
| | 2 | 2 | 0 | 1 | 3 |
| B | 3 | 2 | 0 | 1 | 3 |
| | 4 | 2 | 0 | 1 | 3 |
| C | 5 | 3 | 3 | 1 | 7 |
| | 6 | 3 | 3 | 0 | 6 |
| D | 7 | 2 | 0 | 1 | 3 |
| | 8 | 2 | 0 | 0 | 2 |

[a] Group A, non-vaccinated and challenged group; group B, SL subunit vaccine (HA1 + wtCT); group C, IM subunit vaccine (HA1 + aluminum hydroxide); and group D, IM inactivated vaccine (+ aluminum hydroxide).
[b] Criteria for lung scoring: Severe, 3; Moderate, 2; Mild, 1; No sign, 0

were (6, 3), (3, 3), (7, 6), and (3, 2) in groups A, B, C, and D, respectively (Table 4). The pig with highest lung score (7) was found in group C, while the lowest (2) in Group D. Representative gross lesions of each group are presented in Fig. 2 (panels a, d, g, and j). Virus-specific lesions were characterized by focal hepatisation with clear demarcation from normal lung regions. All groups showed moderate to severe interstitial pneumonia (Figs. 2b, 2e, 2h, and 2k) with inter-individual variation. While moderate to severe bronchial pneumonia was observed only in groups A and C, mild suppurative tracheitis was found in all groups (Figs. 2c, 2f, 2i, and 2l).

## Discussion

Since pigs are engaged in the pandemic influenza virus outbreak in humans, experimental pandemic influenza virus-based vaccines were tested in pigs. The pigs are physiologically and anatomically similar to human, which can provide more practical information than rodents in the influenza vaccine study. Yucatan miniature pigs were selected for the study because they are easy to handle and pure breed. However, since the number of Yucatan pigs was limited, two of each group were just available in this study. Despite of this limitation, the data showed some differences among groups and gave possibilities for the large-scale of the influenza vaccine studies using pigs.

Recently, SL vaccination of inactivated influenza A virus was effective in inducing systemic and mucosal immune responses in mice, when co-administered with mCTA/LTB, a subunit of mutant cholera toxin (CT) E112K with the pentameric B subunit of a heat-labile enterotoxin from enterotoxigenic *E. coli* (LT) (Song *et al.*, 2008b). However, in this study, wtCT-based pandemic influenza virus vaccine (subunit HA1 protein) was administered, since wtCT is also known to induce systemic and mucosal immune responses (Elson, 1989; Lycke *et al.*, 1992). Therefore, in this study, vaccination via the SL route with HA1 protein plus wtCT was first tried in pigs and compared to IM administration of inactivated whole virus and HA1 protein with aluminum hydroxide as an adjuvant.

As expected, the conventional vaccination protocol (IM administration of inactivated whole virus vaccine with the adjuvant, aluminum hydroxide) showed good efficacy. It

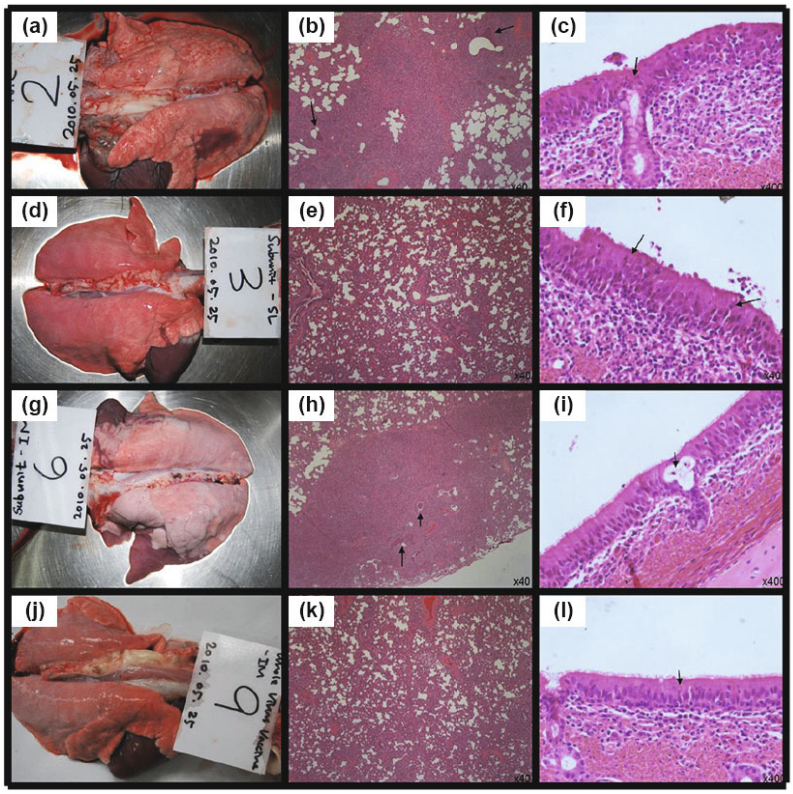


**Fig. 2. Pathological findings in the different vaccination groups 2 weeks after the challenge.** Group A, non-vaccinated and challenged group; group B, SL subunit vaccine (HA1 + wtCT); group C, IM subunit vaccine (HA1 + aluminum hydroxide); and group D, IM inactivated vaccine (+ aluminum hydroxide). a, d, g, j, gross lesions of lungs in groups A, B, C, and D, respectively; b, e, h, k, histopathology of lungs (40×) in groups A, B, C, and D, respectively; c, f, i, l, histopathology of trachea (400×) in groups A, B, C, and D, respectively. The arrows indicates neutrophil infiltration in bronchiolar lumens and tracheal epithelium.

could induce the highest HI titre and reduce the quantity and duration of viral shedding as well as lung lesion formation. SL administration of subunit (HA1 protein with wtCT) vaccination also reduced histopathological lesions compared to the non-vaccinated pigs. Reduced viral shedding and priming for humoral immune response were also observed. However, HI titres induced by sublingually administered HA1 protein plus wtCT was not as high as the conventional vaccination protocol. Since CT is known as an effective mucosal adjuvant (Elson, 1989; Lycke *et al*., 1992), these findings indicated that SL administration of subunit (HA1 protein with wtCT) vaccine protected pigs from pandemic influenza by a different immune mechanism than that of the conventional vaccination system (IM administration of inactivated whole virus vaccine with the adjuvant, aluminum hydroxide).

Interestingly, when the HA1 protein was intramuscularly administered with aluminum hydroxide, no obvious reduction of viral shedding and lung lesions were found. Nonetheless, evidence of priming for humoral immune responses was observed. These results meant that subunit vaccine with aluminum hydroxide via the IM route did not fully protect pigs from the challenge. This result is consistent with a previous report stating that inactivated influenza vaccine is superior to subunit vaccine in mice immunized through IM vaccination (Geeraedts *et al*., 2008). Since recombinant HA1 protein in this study was prepared by using the classical bacterial expression system, posttranscriptional changes required for its immunogenicity might not have occurred. However, recent studies revealed that the proper folding method or fusion with ligand for TLRs could induce protective immunity to animals, when the recombinant protein produced by bacterial expression system was used (Aguilar-Yanez *et al*., 2005; Song *et al*., 2008a). Therefore, in the present study, the insufficient protection observed in the pigs intramuscularly vaccinated with HA1 protein plus aluminum hydroxide might be related to improper folding of protein structure.

Nevertheless, the pigs vaccinated with HA1 protein plus wtCT via the SL route were thought to be protected from the challenge. The differences of adjuvant and administration route may also be associated with the vaccine efficacy of the HA1 protein in addition to the proper folding or glycosylation. Since aluminum hydroxide-based adjuvant system was more effective in inducing $Th_2$ immune reaction rather than Th1 immune reaction (Petrovsky and Aguilar, 2004), the humoral immune response from a single protein (HA1) was not enough to protect pigs from pandemic influenza infection. In contrast, the wtCT was also known to induce systemic and mucosal immune responses (Elson, 1989; Lycke *et al*., 1992). Moreover, SL administration of influenza virus vaccines has proved to be effective for mucosal and systemic immune responses in mice (Song *et al*., 2008b). Therefore, wtCT-based adjuvant and SL administration could be an effective way of inducing protective immunity in pigs. In this study, we provided evidence for the effectiveness of SL administration of subunit pandemic influenza vaccine (HA1 protein + wtCT) in protecting pigs from pandemic influenza. Although HA1 protein from bacterial expression system was used, the protective effect was as good as the conventional vaccination method. However, the underlying immune mechanism appeared different. Since pandemic influenza virus (H1N1) used in this study was reported to be close to the swine H1N1 influenza virus (Garten *et al*., 2009), the findings of this study might be of help for further investigation on the use of SL vaccination in humans. Furthermore, recent studies which demonstrated the protective immunity in mice through SL administration of subunit or live attenuated influenza vaccine gives higher possibility of SL administration in human application (Shim *et al*., 2011; Park *et al*., 2012).

In this study, SL administration of HA1 subunit vaccine (with wtCT adjuvant) was tested for its efficacy in pigs. Due to the limited number of experimental pigs, this study cannot provide detailed comparative information about what type of vaccines or administration route are better, but can provide the protective potential of SL administration for influenza vaccination in pigs. There should be additional animal studies about effective adjuvant system and vaccine types for the use of SL influenza vaccination comparing diverse pathogenic markers for influenza virus infection.

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