



Title	Mucosal vaccination against influenza : Protection of pigs immunized with inactivated virus and ether-split vaccine
Author(s)	LIM, Yoon-Kyu; TAKADA, Ayato; TANIZAKI, Takashi; OZAKI, Hiroichi; OKAZAKI, Katsunori; KIDA, Hiroshi
Citation	Japanese Journal of Veterinary Research, 48(4): 197-203
Issue Date	2001-02-28
DOI	10.14943/jjvr.48.4.197
Doc URL	<a href="http://hdl.handle.net/2115/2860">http://hdl.handle.net/2115/2860</a>
Type	bulletin
File Information	KJ00002400316.pdf



[Instructions for use](#)

## **Mucosal vaccination against influenza : Protection of pigs immunized with inactivated virus and ether-split vaccine**

**Yoon-Kyu Lim, Ayato Takada, Takashi Tanizaki, Hiroichi Ozaki, Katsunori Okazaki, Hiroshi Kida<sup>1\*</sup>**

(Accepted for publication : January 29, 2001)

### **Abstract**

Effective vaccinations against swine influenza reduce the economic loss of pig industries, and also may minimize the possibility of emergence of new pandemic viruses, since pigs are intermediate hosts to generate reassortant viruses among avian and mammalian influenza viruses. In this study, we showed that intranasal immunization of pigs with formalin-inactivated or ether-split influenza vaccine (A/Aichi/2/68) induced virus-specific IgG, IgM, and IgA antibodies in their nasal secretions and sera, resulting in complete protection from virus challenge. Antibody response to the challenge virus was not observed in the immunized pigs, suggesting that the replication of the virus in the primary targets, respiratory epithelial cells, was inhibited. The present results indicate that intranasal immunization of pigs with inactivated vaccines is effective to control swine influenza, and also provide a good model, as well as a mouse model, to evaluate an intranasal application of influenza vaccine for humans.

### **Introduction**

Swine influenza is a highly contagious acute viral disease of the respiratory tract, and has economic consequences in that infected pigs lose weight and occasionally die by

secondary infections<sup>3)</sup>. In addition to the significance of this disease with economic loss of pig industries, there is, in fact, another issue that should be considered with control of swine influenza.

Pigs are shown to be susceptible to both

---

Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

\*To whom correspondence should be addressed.

H. Kida : Department of Disease Control, Graduate School of Veterinary Medicine, Hokkaido University, Sapporo 060-0818, Japan

Phone : +81-11-706-5207, Fax : +81-11-706-5273, e-mail : kida@vetmed.hokudai.ac.jp

Y. -K. Lim, present address : Department of Veterinary Medicine, Cheju National University, Cheju 690-756, Korea

A. Takada, present address : Department of Microbiology and Immunology, The Institute of Medical Science, The University of Tokyo, Tokyo 108-8639, Japan

human and avian influenza A viruses<sup>2,5,8</sup>), which may be explained by the existence of the receptors for both viruses on the epithelial cells of their respiratory tracts<sup>6</sup>). The human pandemic strain, A/Hong Kong/2/68 (H3N2), has been shown to be a genetic reassortant between human and avian viruses, and such an event might occur in pigs coinfecting with the viruses<sup>22</sup>). Thus, pigs are thought to be intermediate hosts playing an important role to generate human pandemic influenza viruses<sup>14,22</sup>).

The influenza vaccines that are commercially available for animals, including humans, horses, and pigs do not provide complete protection from virus infection while they may decrease the incidence and severity of clinical disease<sup>12</sup>). These vaccines are inactivated intact virus or subunit vaccines, and injected by parenteral route to induce serum antibody response. On the other hand, there is a large number of works showing that the mucosal immunity represented by local secretory antibodies such as IgA, is rather more essential for protection of animals from infections of mucosal tissues than systemic immunity<sup>10,11</sup>). Since the secretory antibodies play an important role to exclude viruses from the mucosal surfaces of animals, induction of the secretory antibodies may provide complete protection from virus infections through the mucosal routes by prohibiting their initial replication in the mucosal epithelial cells<sup>17,18,20</sup>).

Influenza virus initially infects the epithelial cells of the respiratory tracts of the animals. Accordingly, it has been shown that the local secretory antibodies play an important role for the protection of mice from the virus infection<sup>4,19,21</sup>). The aim of this study is to evaluate intranasal vaccination of pigs with inactivated influenza vaccines, which have a potential to induce secretory antibodies and hence to elicit protective immunity in the ani-

mals.

## Materials and Methods

### *Animals*

Three-week-old specific pathogen free pigs (F1 between Landrace and Durock) were purchased from Hokuren (Sapporo, Japan).

### *Virus and vaccines*

Influenza virus, A/Aichi/2/68 (H3N2) (Aichi/2/68), a prototype strain of human H3N2 viruses, was propagated in the allantoic cavities of 11-day-old embryonated chicken eggs at 35°C for two days. The virus was purified by high speed centrifugation of infected allantoic fluid, followed by differential centrifugation through a 10 to 50% sucrose density gradient and pelleted<sup>9</sup>). For ether-split vaccine, the purified virus was mixed with equal volume of ether, followed by incubation for 30 min at room temperature with stirring. The mixture was centrifuged (3,000rpm, 15 min), and the aqueous phase was collected and evaporated. Formalin was added to the aqueous phase at the final concentration of 0.01%. For inactivated intact virus vaccine, formalin was added to the allantoic fluid and then the virus was purified as described above.

### *Immunization and protection tests*

Pigs were immunized intranasally with 100µg viral protein of each vaccine diluted with 1 ml of PBS (0.5ml for each nostril) 4 times weekly. Control pigs were given PBS. One week after the final immunization, the sera and nasal swabs were collected for antibody detection, and the pigs were challenged intranasally with 1 ml of 10<sup>4</sup> plaque forming units (PFU) of the virus. To examine virus recovery, the nasal swabs were collected from the pigs daily after the challenge. The swabs were soaked into 1 ml of PBS containing antibiotics. Virus titers were determined by

plaque assays using MDCK cells as described previously<sup>7)</sup>.

#### Antibody assays

Sera and nasal swabs were examined for their antibody titers by hemagglutination-inhibition (HI) test<sup>15)</sup> and enzyme-linked immunosorbent assay (ELISA)<sup>7)</sup>. For HI tests, the sera were treated with receptor destroying enzyme (Takeda Chemical Industries) to reduce nonspecific HI activity by serum inhibitors. In the ELISA, the wells were coated with disrupted Aichi/2/68 obtained by treating purified virions with 0.05M Tris-HCl (pH 7.8) containing 0.5% Triton X-100 and 0.6M KCl at room temperature and diluted in PBS. The reactions were detected using goat anti-pig IgA, IgM, or IgG antibodies conjugated to horseradish peroxidase (Bethyl Laboratories).

### Results

#### Antibody response of pigs immunized intranasally with formalin-inactivated virus or ether-split vaccine

Four and two pigs were immunized intranasally with formalin-inactivated virus and ether-split vaccines, respectively. One week after the final immunization, sera and nasal swab samples were collected from these immunized pigs and 4 control pigs, and examined for their antibody responses (Table 1). We found that both formalin-inactivated virus and ether-split vaccines induced systemic and mucosal antibody responses. Remarkable levels of IgG, IgM, and IgA antibodies specific to the virus were detected both in the sera and nasal secretions of the immunized pigs. No significant difference in the ability to induce systemic and mucosal antibody responses was observed between formalin-inactivated virus and ether-split vaccines.

To monitor the antibody levels in the immunized pigs during the experiments, serum and nasal swab samples were collected from each two of four pigs in the groups I (formalin-inactivated vaccine) and III (control) weekly after the first immunization, and IgG and IgA antibody titers in the sera and the nasal secretions, respectively, were determined

Table 1. Virus-specific antibody titers<sup>a</sup> of pigs immunized intranasally with inactivated vaccines.

group	pig no.	serum			nasal swab		
		IgG	IgM	IgA	IgG	IgM	IgA
I. formalin-inactivated vaccine	1	800	200	400	320	320	640
	2	800	200	400	160	160	160
	3	640	NT <sup>b</sup>	NT	160	NT	160
	4	640	NT	NT	160	NT	640
II. enter-split vaccine	5	800	400	200	160	160	160
	6	1600	200	400	160	80	640
III. control (PBS)	7	<10	<10	<10	<10	<10	<10
	8	<10	<10	<10	<10	<10	<10
	9	<10	<10	<10	<10	<10	<10
	10	<10	<10	<10	<10	<10	<10

<sup>a</sup> IgG, IgM, and IgA antibodies in the samples of individual pigs were detected by ELISA as described in the Materials and Methods. Antibody titers are expressed as the reciprocals of end-point dilution of the samples.

<sup>b</sup> Not tested.

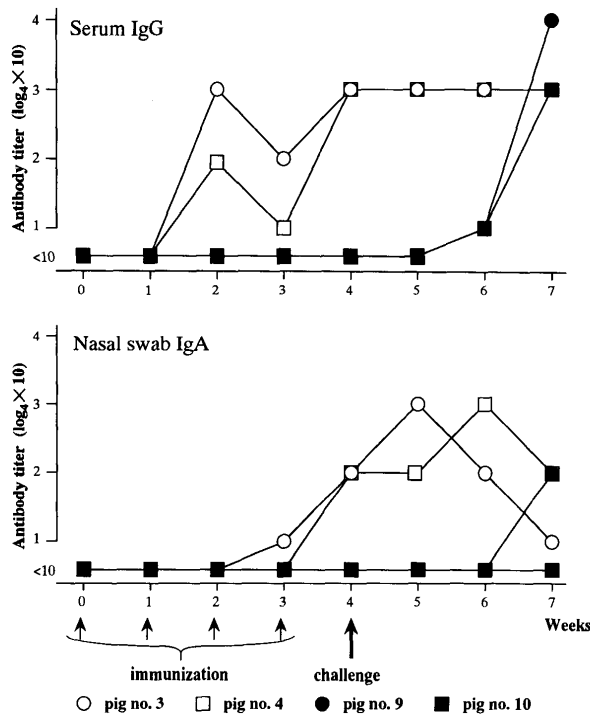


Fig. 1. Serum IgG and nasal IgA antibody responses in pigs after immunization and virus challenge. Samples were collected from pigs nos. 3 and 4 that were immunized with formalin-inactivated virus vaccine, and control pigs no. 9 and 10 every week. Antibody titers were determined by ELISA.

(Fig. 1). Both of the immunized pigs (nos. 3 and 4) produced detectable serum IgG antibodies after the second immunization, while nasal IgA antibodies became positive after the third or fourth immunization. One week after the final immunization, antibody titers of serum IgG and nasal IgA reached up to 640 and 160, respectively.

#### Protection of pigs from intranasal virus challenge

To evaluate the contribution of the antibodies to protective effects, pigs were challenged with Aichi/2/68 at one week after the final immunization. Nasal swab samples were collected daily for 10 days and examined for virus recovery (Table 2). On 2-7 days after the virus challenge, control pigs (group III) shed the virus at the titers of  $10^{1.3}$ - $10^{2.9}$  PFU/ml of samples. In contrast, no virus was detected in the samples of two pigs in group II (pigs nos. 5 and 6) and two out of four pigs in group I (pigs nos. 1 and 2) throughout the periods. Only a low level of the virus was recov-

Table 2. Virus recovery from the nasal swabs of pigs immunized intranasally.

group	pig no.	days post infection <sup>a</sup>										
		0	2	3	4	5	6	7	8	9	10	
I	1	— <sup>b</sup>	—	—	—	—	—	—	—	—	—	—
	2	—	—	—	—	—	—	—	—	—	—	
	3	—	—	1.5 <sup>c</sup>	—	—	—	—	—	—	—	
	4	—	—	1.5	—	—	—	—	—	—	—	
II	5	—	—	—	—	—	—	—	—	—	—	
	6	—	—	—	—	—	—	—	—	—	—	
III	7	—	1.8	2.9	2.5	2.8	2.3	—	—	—	—	
	8	—	2.0	2.8	2.2	2.7	1.3	—	—	—	—	
	9	—	1.9	2.0	1.8	2.9	2.9	2.1	—	—	—	
	10	—	—	2.6	2.5	2.6	1.6	—	—	—	—	

<sup>a</sup> Nasal swabs were collected from each pigs daily after the virus challenge.

<sup>b</sup> — ; not detected.

<sup>c</sup> Titers are expressed as  $\log_{10}$ PFU/ml of swab sample.

Table 3. Serum HI antibody response of pigs immunized intranasally with inactivated vaccines.

group	pig no.	week post infection <sup>a</sup>	
		0	3
I	1	256 <sup>b</sup>	256
	2	256	256
	3	64	64
	4	64	64
II	5	64	64
	6	128	64
III	7	< 8	128
	8	< 8	256
	9	< 8	1024
	10	< 8	512

<sup>a</sup> The sera were collected before and 3 weeks after virus challenge.

<sup>b</sup> Titers are expressed as the reciprocals of serum dilution.

ered from two pigs (pigs no. 3 and 4) in group I at three days after the challenge.

#### *Antibody response of pigs after virus challenge*

In order to know whether the immune response against the challenge virus was induced, serum samples were collected from all the pigs on 0 and 21 days after the challenge and examined for HI antibody titers. As shown in Table 3, no increase in the titers was found in pigs in group I, although pigs nos. 3 and 4 shed the virus, or in those in group II. The control pigs seroconverted after the challenge. In order to examine an effect of protection by inactivated intact virus in detail, serum and nasal swab samples collected from each two of four pigs in groups I (pigs no. 3 and 4) and III (pigs no. 9 and 10) after the virus challenge were tested to compare their antibody titers with those of the samples collected from them before challenge (Fig. 1). In contrast to the control pigs in which significant IgG antibody raise were found, indicat-

ing that the replication of the challenge virus occurred, both of the immunized pigs did not show the increase in antibody titers after the virus challenge. Similarly, significant increase in nasal IgA antibody titers was not observed with the immunized pigs.

#### **Discussion**

Influenza A viruses infect a large variety of animal species<sup>22</sup>. Among them, pigs are an important host in influenza ecology since they are susceptible to infection with both avian and mammalian influenza A viruses. Although only viruses of subtypes H1N1 and H3N2 have been spread widely in pigs thus far<sup>1</sup>, there are many epidemiological evidence that human-to-pig and avian-to-pig transmission could occur in nature<sup>1,23</sup>. It is also noted that avian H9N2 viruses which were isolated from avian species in Southern China were recently isolated from humans and pigs in that area<sup>13,16</sup>. Maintenance of current influenza A viruses in the pig population or the frequent introduction of new viruses from avian species could be a threat of the generation of reassortant viruses which may emerge as pandemic strains in humans. Besides their roles in genetic reassortment, pigs are sometimes sources for zoonotic transmission of swine viruses to humans<sup>12</sup>. Thus, much attention should be paid to the control of swine influenza, not only to reduce the economic loss of pig industries but also to minimize the possible transmission of the viruses, including reassortant viruses, to humans.

Vaccines currently used for animals and humans are injected through parenteral routes (eg. subcutaneous) to induce serum antibodies. However, systemic immunity including serum antibody and cytotoxic T-cell responses does not prevent initial infection on the mucosal epithelial cells<sup>17,18</sup>, although these responses may reduce the severity of

the disease and eliminate the virus from the infected animals<sup>12)</sup>. In contrast, since the mucosal surfaces are the portals of the entry for many pathogens including influenza virus, mucosal immunity protects animals from virus infections by inhibiting initial replication of the virus at the mucosal tissues. In this study, the intranasal immunization of pigs with formalin-inactivated or ether-split influenza vaccine induced virus-specific IgG, IgM, and IgA antibodies in their nasal secretions, resulting in complete protection from the virus challenge. The present results are consistent with our previous studies on intranasal immunization of animals against viral infections<sup>17-20)</sup>.

The present study showed that a complete protection of pigs from influenza A virus infection could be attained by the induction of a specific immune defense at the site of the entry for the virus. This strategy should eliminate any opportunity to generate reassortant viruses in pigs, while systemic immunization does not appear appropriate for preventing an initial replication of the virus in the respiratory tissues. In addition, there is a number of practical advantages for mucosal immunization with non-invasive viral antigens with respect to the simplicity, easy storage and delivery, and cost effectiveness, which should abolish the risks inherent to the use of live virus vaccines.

### Acknowledgements

We thank Hiroki Takakuwa, Masaki Imai, Masato Hatta, Ai Ninomiya, and Tokiko Nagano, for their excellent technical assistance.

### References

- 1) Brown, I. H. 2000. The epidemiology and evolution of influenza viruses in pigs. *Vet. Microbiol.*, 74 : 29-46.
- 2) Chambers, T. M., Hinshow, V. S.,

- Kawaoka, Y., Easterday, B. C., Webster, R. G. 1991. Influenza viral infection of swine in the United States 1988-1989. *Arch. Virol.*, 116 : 261-265.
- 3) Easterday, B. C., Hinshow, V. S. 1992. Swine influenza. In : *Disease of Swine*, pp. 349-357, Leman, A. D. et al., eds., Iowa State Press, Ames.
- 4) el Guink, N., Kris, R. M., Goodman-Snitkoff, G., Small, P. A. Jr., Mannino, R. J. 1989. Intranasal immunization with proteoliposomes protects against influenza. *Vaccine*, 7 : 147-151.
- 5) Hinshow, V. S., Webster, R. G., Easterday, B. C., Bean Jr., W. J. 1981. Replication of avian influenza A viruses in mammals. *Infect. Immun.*, 34 : 354-361.
- 6) Ito, T., Nelson, J., Couceiro, J. N. S. S., Kelm, S., Baum, L. G., Krauss, S., Castucci, M. R., Donatelli, I., Kida, H., Paulson, J. C., Webster, R. G., Kawaoka, Y. 1998. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. *J. Virol.*, 72 : 7367-7373.
- 7) Kida, H., Brown, L. E., Webster, R. G. 1982. Biological activity of monoclonal antibodies to operationally defined antigenic regions on the hemagglutinin molecule of A/Seal/Massachusetts/1/80 (H7N7) influenza virus. *Virology*, 122 : 38-47.
- 8) Kida, H., Ito, T., Yasuda, J., Shimizu, Y., Itakura, C., Shortridge, K. F., Kawaoka, Y., Webster, R. G. 1994. Potential for transmission of avian influenza viruses in pigs. *J. Gen. Virol.*, 75 : 2183-2188.
- 9) Kida, H., Yanagawa, R. 1981. Classification of avian paramyxovirus by immunodiffusion on the basis of antigenic specificity of their M protein antigens. *J. Gen. Virol.*, 52 : 103-111.
- 10) McGhee, J. R. and Mestecky, J. 1990. In

- defense of mucosal surfaces : Development of novel vaccines for IgA responses protective at the portal of entry of microbial pathogens. *Infect. Dis. Clin. North Amer.* , 4 : 315-341.
- 11) Mestecky, J. and Jackson, S. 1994. Reassessment of the impact of mucosal immunity in infection with human immunodeficiency virus (HIV) and design of relevant vaccines. *J. Clin. Immunol.* , 14 : 259-272.
  - 12) Olsen, C. W. 2000. DNA vaccination against influenza viruses : a review with emphasis on equine and swine influenza. *Vet. Microbiol.* , 74 : 149-164.
  - 13) Peiris, M., Yuen, K. Y., Leung, C. W., Chan, K. H., Ip, P. L. S., Lai, R.W., Orr, W. K., Shortridge, K. F. 1999. Human infection with influenza H 9 N2. *Lancet* , 354 : 916-917.
  - 14) Scholtissek, C., Burger, H., Kistner, O., Shortridge, K. F. 1985. The nucleoprotein as a possible major factor in determining host specificity of influenza H 3 N 2 viruses. *Virology* , 147 : 287-294.
  - 15) Sever, J. L. 1962. Application of microtechnique to viral serological investigations. *J. Immunol.* , 88 : 320-329.
  - 16) Shortridge, K. F., Gao, P., Guan, Y., Ito, T., Kawaoka, Y., Markwell, D., Takada, A., Webster, R. G. 2000. Interspecies transmission of influenza viruses : H 5 N 1 virus and a Hong Kong SAR perspective. *Vet. Microbiol.* , 74 : 141-7.
  - 17) Takada, A., Kida, H. 1995. Induction of protective antibody responses against pseudorabies virus by intranasal vaccination with glycoprotein B in mice. *Arch. Virol.* , 140 : 1629-1635.
  - 18) Takada, A., Kida, H. 1996. Protective immune response of chickens against Newcastle disease, induced by the intranasal vaccination with inactivated virus. *Vet. Microbiol.* , 50 : 17-25.
  - 19) Takada, A., Kuboki, N., Okazaki, K., Ni-nomiya, A., Tanaka, H., Ozaki, H., Itamura, S., Nishimura, H., Enami, M., Tashiro, M., Shortridge, K. F., Kida, H. 1999. An avirulent avian influenza virus as a vaccine strain against a potential human pandemic. *J. Virol.* , 73 : 8303-8307.
  - 20) Takada, A., Shimizu, Y., Kida, H. 1994. Protection of mice against Aujeszky's disease virus infection by intranasal vaccination with inactivated virus. *J. Vet. Med. Sci.* , 56 : 633-637.
  - 21) Tamura, S., Samegai, Y., Kurata, H., Nagamine, T., Aizawa, C., Kurata, T. , 1988. Protection against influenza virus infection by vaccine inoculated intranasally with cholera toxin B subunit. *Vaccine* , 6 : 409-413.
  - 22) Webster, R. G., Bean, W. J., Gorman, O. T., Chambers, T. M., Kawaoka, Y. 1992. Evolution and ecology of influenza A viruses. *Microbiol. Rev.* , 56 : 152-179.
  - 23) Zou, N. N., Senne, D. A., Landgraf, J. S., Swenson, S. L., Erickson, G., Rossow, K., Liu, L., Yoon, K. -J., Krauss, S., Webster, R. G. 1999. Genetic reassortant of avian, Swine, and human influenza A viruses in American pigs. *J. Virol.* , 73 : 8851-8856.