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Short communication

A lack of antibody formation against inactivated influenza virus after aerosol vaccination in presence or absence of adjuvantia

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ABSTRACT

In the poultry industry, infections with avian influenza virus (AIV) can result in significant economic losses. The risk and the size of an outbreak might be restricted by vaccination of poultry. A vaccine that would be used for rapid intervention during an outbreak should be safe to use, highly effective after a single administration and be suitable for mass application. A vaccine that could be applied by spray or aerosol would be suitable for mass application, but respiratory applied inactivated influenza is poorly immunogenic and needs to be adjuvanted. We chose aluminum OH, chitosan, cholera toxin B subunit (CT-B), and Stimune as adjuvant for an aerosolized vaccine with inactivated H9N2. Each adjuvant was tested in two doses. None of the adjuvanted vaccines induced AIV-specific antibodies after single vaccination, measured 1 and 3 weeks after vaccination by aerosol, in contrast to the intramuscularly applied vaccine. The aerosolized vaccine did enter the chickens' respiratory tract as CT-B-specific serum antibodies were detected after 1 week in chickens vaccinated with the CT-B-adjuvanted vaccine. Chickens showed no adverse effects after the aerosol vaccination based on weight gain and clinical signs. The failure to detect AIV-specific antibodies might be due to the concentration of the inactivated virus.

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1. Introduction

In the poultry industry, infections with avian influenza virus (AIV) can result in significant economic losses. The risk and the size of an outbreak of AIV could be restricted by vaccination of poultry. Possible vaccination strategies are rapid intervention (emergency vaccination of poultry in the area around an outbreak), preventive vaccination of specific categories of poultry that are more at risk for a new introduction of AIV (e.g. free range layers in areas with many wild ducks and geese), and general preventive vaccinations of poultry in areas in which AIV is endemic. A vaccine used for rapid intervention during an outbreak should be safe, highly effective after a single administra-

tion and be suitable for mass application. A vaccine that could be applied by spray or aerosol would be suitable for mass application, which is regularly performed for e.g. infectious bronchitis virus (De Wit et al., 2010), Newcastle disease virus (NDV), avian metapneumovirus and most *Mycoplasma gallisepticum* vaccines (Ley, 2003). In the case of AIV, aerosol vaccination using live virus is not desirable because of its zoonotic potential and because of the risk for virus reassortment. Mucosal vaccination via the respiratory route has several advantages: it induces both local and systemic immune responses (Atmar et al., 2007; Tseng et al., 2009; Worrall et al., 2009), it could halt infection already at portal of entry (Yoshikawa et al., 2004) and it is suitable for mass application.

The respiratory tract (RT) mucosa constantly comes into contact with inhaled Ag and in normal circumstances these Ag do not provoke strong immune responses, but induce a state of tolerance (Akbari et al., 2001) leading to a tolerogenic environment in the RT. Intranasally (i.n.) applied whole inactivated AIV (WIV) is poorly immunogenic (Hagenaars et al., 2008), as was also described in chicken for i.n. applied WIV (Worrall et al., 2009) and i.n. applied inactivated NDV (Tseng et al., 2009). To enhance the immunogenicity of WIV it needs to be adjuvanted. We chose aluminum (alum) OH, chitosan, cholera toxin B subunit (CT-B), and Stimune as adjuvant for in an aerosolized vaccine with inactivated H9N2. Alum adjuvants are the most widely used adjuvants for human vaccines (Lambrecht et al., 2009) and it is an effective adjuvant in influenza vaccines in mice (Chang et al., 2010) and chicken (Reemers et al., 2010). However, in human influenza trials results are less consistent, with an aluminum phosphate adjuvanted H9N2 WIV i.m. vaccine being well tolerated and immunogenic (Nicholson et al., 2009), while other groups found that H1N1 split virus (detergent-disrupted virion) vaccine formulations containing alum were less immunogenic than formulations without adjuvant when given i.m. (Zhu et al., 2009). Chitosan has shown promising results in i.n. split virus vaccines in mice (Bacon et al., 2000), split virus and protein vaccines in human (Read et al., 2005; Sui et al., 2010a,b) and a split virus vaccine in poultry (Worrall et al., 2009; Rauw et al., 2010). It was previously shown that CT is an effective mucosal adjuvant in chicken (Vervelde et al., 1998), but CT cannot be used in the field because of its toxicity. CT-B containing trace amounts of CT is an effective adjuvant in i.n. delivered split virus vaccines in mice (Matsuo et al., 2000), but because of the toxicity of CT and the mass application as intended in the field, we decided to use pure CT-B. CT-B already showed protective effects in chickens with an i.n. applied inactivated NDV vaccine (Takada and Kida, 1996). Stimune, also known as Specol, has been used successfully in an i.m. vaccine using soluble trimeric H5 protein in chicken (Cornelissen et al., 2010).

In this study, different adjuvants were tested in an aerosolized vaccine using WIV for use in a rapid intervention strategy. None of the adjuvanted vaccines induced AIV-specific Ab after a single vaccination, measured 1 and 3 weeks post vaccination (wpv) by aerosol, in contrast to the i.m. applied vaccine. The aerosolized vaccine did enter the chickens' RT as CT-B-specific Ab were detected in serum

from 1 wpv onwards. Chickens showed no adverse effects on weight gain and no clinical signs after aerosol vaccination.

2. Materials and methods

2.1. Chickens

One-day-old specific pathogen free broiler chickens (a crossbred of Hybro and Cobb) of both sexes (Animal Health Service, Deventer, the Netherlands) were housed at the Utrecht University animal facilities. Animals were housed in groups on the floor with sawdust bedding and received food and water ad libitum. Aerosol vaccination was performed at Animal Health Service Deventer. In compliance with Dutch law, all experiments were approved by the Animal Experimental Committee of the Faculty of Veterinary Medicine of Utrecht University, the Netherlands, in accordance with the Dutch regulation on experimental animals.

2.2. Virus

H9N2 A/Chicken/Saudi Arabia/SP02525/3AAV/2000 (Animal Health Service, Deventer, the Netherlands) was used for vaccination. The virus titer was 1.2×10^8 EID50/ml or 405 haemagglutinating units (HAU)/ml. Before vaccination, the virus was inactivated using beta-propiolactone (BPL; Acros organics). Briefly, a 10% BPL solution was prepared in a 125 mM sodium citrate and 150 mM sodium chloride buffer and 10 μl/ml was added to the virus. Virus-BPL solution was then incubated for 24 h at 4 °C under continuous stirring. Inactivation was confirmed by inoculation in embryonated chicken eggs performed by Animal Health Service Deventer.

2.3. Vaccines

Each adjuvant was used in 2 doses: a low dose and a high dose. Imject alum (Pierce) was used 1:3 and 1:1 mixed with WIV. Stimune (Prionics) was mixed 1:2 and 1:1 for i.m. vaccination, Stimune was mixed with WIV according to manufacturer's instructions (5 parts Stimune and 4 parts water phase). CT-B (Sigma) was used at a concentration of 10 μg/chicken and 50 μg/chicken. Chitosan (Protasan UP CL 213; Novamatrix) was used as a 1.5% (w/v) and a 3% (w/v) solution in sterile saline.

Chickens were vaccinated with approximately 75 HAU WIV. To calculate the amount of virus needed, the breathing volume was estimated at 44 l/kg body weight per hour (Fedde et al., 1998) and the volume of the isolator was 1.38 m³. The i.m. control group was vaccinated with 700 μl containing 75 HAU WIV adjuvanted with Stimune.

2.4. Experimental set-up

Ninety-five three-week-old SPF broilers were randomized into 9 groups of 10 chickens and one control group of 5 chickens. Group 1 was aerosol-vaccinated with WIV only; groups 2 and 3 were aerosolized with alum-adjuvanted vaccine in a low and high dose respectively; groups 4 and 5 were treated with chitosan-adjuvanted vaccine in a low

and high dose; groups 6 and 7 were treated with the CT-B-adjuvanted vaccine in low and high dose; groups 8 and 9 were aerosolized with the Stimune-adjuvanted vaccine and group 10 was i.m. vaccinated with Stimune-adjuvanted vaccine.

At the age of 3 weeks chickens were vaccinated by aerosol. Animals were vaccinated in groups in isolators (Beyer & Eggelaar) with a volume of 1.38 m^3 (1.94 m long, 0.75 m wide and 0.95 m high). The vaccine was aerosolized using a Walther Pilot I spray-head with 0.5 mm nozzle (Walther Spritz- und Lackiersysteme), as described previously (Corbanie et al., 2008). After aerosolizing the vaccine, chickens were left for 1 h in the isolator to inhale the vaccine. To check whether any of the aerosolized vaccines had adverse effects, chickens were weighed weekly and were checked for adverse effects on eyes and RT right after vaccination and in the week following vaccination. Blood was collected before and 1 and 3 wpv and tracheal swabs were taken 3 wpv. Chickens were killed 3 wpv.

2.5. Tracheal swabs

Swabs were put in 350 μl PBS and mixed for 1 h at room temperature to elute tracheal swab content. Swabs fluid was used for ELISA.

2.6. Avian influenza virus ELISA

IDEXX FlockChek Avian Influenza MultiS-Screen Ab Test Kit (IDEXX) was used according to manufacturer's instructions. This ELISA is based on a competitive blocking approach, the specific sample antibodies block the enzyme-labeled, specific antibody in the conjugate. The addition of an enzyme substrate-chromogen reagent causes color to develop. This color is inversely proportional to the amount of bound sample antibody. The more antibodies present in the sample, the less color development in the test wells. Sample/negative (S/N) ratio was calculated as follows: S/N ratio = sample absorbance/negative control absorbance.

AIV-specific Ab were determined in serum and in tracheal swab samples. Serum samples were diluted according to manufacturer's instructions. Tracheal swab elutes were used without diluting.

2.7. CT-B antibody ELISA

To detect CT-B-specific Ab, a ganglioside M (GM)-1 ELISA was performed as described previously by Stok et al. (1994). Briefly, high bind microplates (Corning) were coated with 2 μg/ml GM-1 (Sigma) at 4 °C overnight. Then 0.2 μg/well CT-B (Sigma) was added and plates were incubated for 1 h at room temperature. Serum was added starting at a dilution of 1:50 and 1:1 serially diluted to 1:6400 and incubated for 1 h at 37 °C. Horseradish peroxidase-labeled goat-anti chicken IgG (H + L; Southern Biotech) was added and plates were incubated for 1 h at room temperature. Plates were developed using one-step ultra TMB (Pierce). Color development was stopped using 1 M H_2SO_4 and extinction was measured at 450 nm.

3. Results and discussion

In this study, inactivated AIV antigen with different adjuvants was tested in an aerosolized vaccine for use in a rapid intervention strategy. A vaccine used for rapid intervention vaccination strategies is aimed at stopping an ongoing outbreak. It should therefore induce a rapid protection, ideally after a single vaccination by a mass application method to be effective in the field situation. Aerosol vaccinations were performed in an isolator, using a Walther Pilot I spray-head. This set-up was used in a previous experiment to aerosolize NDV (Corbanie et al., 2008) and the spray-head was used previously to aerosolize fluorescent microspheres (Corbanie et al., 2006). It was found that using this set-up the concentration of NDV in the isolator did not decrease when measured up until 20 min after nebulization (Corbanie et al., 2008) and fluorescent microspheres were present throughout the respiratory tract, including air sacs, at 20 min after administration (Corbanie et al., 2006). Furthermore, in a previous experiment we performed aerosol inoculation with live H9N2 AIV (Reemers et al., 2009). In another study chickens were inoculated with virus via the intratracheal route and found similar immune responses and similar kinetics of the responses (Rebel et al., 2011).

Chickens were aerosol-vaccinated at 3 weeks of age with H9N2 WIV adjuvanted with either alum, chitosan, CT-B or Stimune. To determine if the adjuvants had any adverse effects on weight gain of the chickens, birds were weighed weekly. To adjust for growth differences between the sexes, we set the pre-vaccination body weight (day 19) at 100% for each individual chicken and used this to calculate relative body weights. We found no significant differences in relative body weight between any of the treatment groups (data not shown). Furthermore, we did not observe any respiratory or eye problems right after vaccination and in the weeks following vaccination. The vaccines were therefore safe to use.

From previous experiments using an i.m. applied vaccine, it was determined that 42–128 HAU were needed for an efficient protection against a high-pathogenic H7N7 infection (Maas et al., 2009). In our experiments, chickens were vaccinated with approximately 75 HAU by aerosol. Chickens were bled before and 1 and 3 wpv to detect AIV-specific serum Ab after the vaccination.

The presence of AIV-specific Ab in serum at 1 and 3 wpv was determined using a well validated ELISA. In this commercial ELISA, the samples containing AIV-specific antibodies have a sample/negative (S/N) ratio <0.5. Pre-vaccination, no AIV-specific Ab were present in serum. From 1 wpv, AIV-specific serum Ab were detected in the i.m. vaccinated group, but not in any of the aerosol-vaccinated animals (Fig. 1).

Primary i.n. vaccination in chicken with 100 HAU split H5N1 virus + sialidase and 0.5% (w/v) chitosan already induced mucosal AIV-specific IgA responses at 1 wpv as determined in tracheal swab elutes (Worrall et al., 2009). When the antigen was given with chitosan alone, AIV-specific IgA responses were lower and IgA was not detected at all time points (Worrall et al., 2009). When chickens were vaccinated at 1 day of age with replication-competent
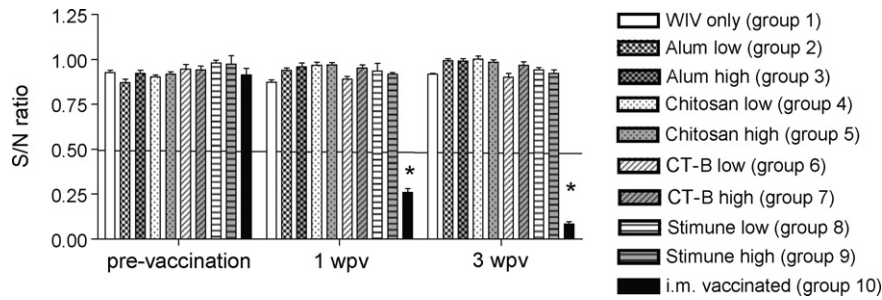
Fig. 1. AIV-specific Ab in serum of aerosol-vaccinated chickens. Presence of AIV-specific Ab in serum was determined pre-vaccination, 1 wpv and 3 wpv using the IDEXX multispecies AIV antibody ELISA. Data are shown as mean S/N ratio + SEM. AIV-specific serum antibodies are detected in samples with S/N ratios <0.5. *$P < 0.05$ for S/N ratio when compared to WIV only.

adenovirus-free AIV H7 vaccine via coarse spray, no HI titers were detected in serum, however AIV-specific IgA was detected in tears at day 10 post vaccination (Toro et al., 2010). To test whether in our experiment Ab were locally induced following aerosol vaccination, AIV-specific Ab were determined in tracheal swab elutes. None of the samples contained detectable levels of influenza-specific Ab (data not shown).

To test if the aerosolized vaccine had entered the RT and had induced immune responses, a CT-B antibody ELISA was performed using sera of chickens vaccinated with CT-B-adjuvanted vaccines. Pre-vaccination no CT-B-specific Ab were detected. When the low dose of CT-B (10 μg/chicken) was given, CT-B-specific Ab were detected at 3 wpv. However, the high dose CT-B (50 μg/chicken) already induced CT-B-specific Ab at 1 wpv and Ab titers were increased at 3 wpv. Vaccination with the high dose CT-B significantly increased the titer of CT-B specific Ab as compared to the low dose of CT-B (Fig. 2).

In summary, although the aerosolized vaccines did enter the chicken RTs, either the dose of influenza virus used was insufficient or a booster vaccination would be needed in order to induce detectable levels of Ab. It has been reported in different mouse studies that i.n. vaccination with adjuvanted WIV (Joo et al., 2010) or split viruses (Ichinohe et al., 2005; Ichinohe et al., 2006; Saluja et al., 2010) requires a boost vaccination for effective induction of Ab. Furthermore, in chicken LPS-containing liposomal inactivated NDV vaccine also required a booster vaccination for

effective induction of serum IgG titers (Tseng et al., 2009). As we already observed CT-B-specific antibodies in serum at 1 wpv, the aerosol vaccination technique is in principle feasible to use in a rapid intervention strategy. The number of HAU needed for protection was determined from i.m. applied vaccine and therefore in a future experiment we will increase the dose in order to determine the number of HAU needed in a respiratory applied vaccine.

Conflict of interest

Acknowledgements

References

Akbari, O., DeKruyff, R.H., Umetsu, D.T., 2001. Pulmonary dendritic cells producing IL-10 mediate tolerance induced by respiratory exposure to antigen. Nat. Immunol. 8, 725–731.

Atmar, R.L., Keitel, W.A., Cate, T.R., Munoz, F.M., Ruben, F., Couch, R.B., 2007. A dose–response evaluation of inactivated influenza vaccine given intranasally and intramuscularly to healthy young adults. Vaccine 29, 5367–5373.

Bacon, A., Makin, J., Sizer, P.J., Jabbal-Gill, I., Hinchcliffe, M., Illum, L., Chatfield, S., Roberts, M., 2000. Carbohydrate biopolymers enhance antibody responses to mucosally delivered vaccine antigens. Infect. Immun. 68 (10), 5764–5770.

Chang, H., Li, X., Teng, Y., Liang, Y., Peng, B., Fang, F., Chen, Z., 2010. Comparison of adjuvant efficacy of chitosan and aluminum hydroxide for intraperitoneally administered inactivated influenza H5N1 vaccine. DNA Cell Biol. 9, 563–568.

Corbanie, E.A., Matthijs, M.G., van Eck, J.H., Remon, J.P., Landman, W.J., Vervaet, C., 2006. Deposition of differently sized airborne microspheres in the respiratory tract of chickens. Avian Pathol. 6, 475–485.

Corbanie, E.A., Vervaet, C., van Eck, J.H., Remon, J.P., Landman, W.J., 2008. Vaccination of broiler chickens with dispersed dry powder vaccines as an alternative for liquid spray and aerosol vaccination. Vaccine 35, 4469–4476.

Cornelissen, L.A., de Vries, R.P., de Boer-Luijtze, E.A., Rigter, A., Rottier, P.J., de Haan, C.A., 2010. A single immunization with soluble recombinant

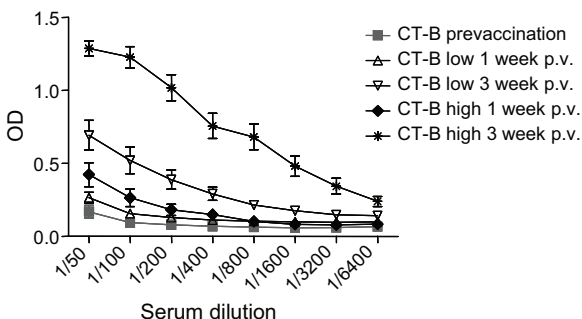

Fig. 2. CT-B-specific Ab in serum of chickens aerosol-vaccinated with CT-B adjuvanted vaccine. The presence of CT-B-specific Ab in serum was determined pre-vaccination, 1 wpv and 3 wpv. Data are shown as mean OD ± SEM.

trimeric hemagglutinin protects chickens against highly pathogenic avian influenza virus H5N1. PLoS One 5, e10645.

De Wit, J.J., Swart, W.A., Fabri, T.H., 2010. Efficacy of infectious bronchitis virus vaccinations in the field: association between the alpha-IBV IgM response, protection and vaccine application parameters. Avian Pathol. 2, 123–131.

Fedde, M.R., Weigle, G.E., Wideman Jr., R.F., 1998. Influence of feed deprivation on ventilation and gas exchange in broilers: relationship to pulmonary hypertension syndrome. Poult. Sci. 11, 1704–1710.

Hagenaars, N., Mastrobattista, E., Glansbeek, H., Heldens, J., van den Bosch, H., Schijns, V., Betbeder, D., Vromans, H., Jiskoot, W., 2008. Head-to-head comparison of four nonadjuvanted inactivated cell culture-derived influenza vaccines: effect of composition, spatial organization and immunization route on the immunogenicity in a murine challenge model. Vaccine 26, 6555–6563.

Ichinohe, T., Watanabe, I., Ito, S., Fujii, H., Moriyama, M., Tamura, S., Takahashi, H., Sawa, H., Chiba, J., Kurata, T., Sata, T., Hasegawa, H., 2005. Synthetic double-stranded RNA poly(I:C) combined with mucosal vaccine protects against influenza virus infection. J. Virol. 5, 2910–2919.

Ichinohe, T., Watanabe, I., Tao, E., Ito, S., Kawaguchi, A., Tamura, S., Takahashi, H., Sawa, H., Moriyama, M., Chiba, J., Komase, K., Suzuki, Y., Kurata, T., Sata, T., Hasegawa, H., 2006. Protection against influenza virus infection by intranasal vaccine with surf clam microparticles (SMP) as an adjuvant. J. Med. Virol. 7, 954–963.

Joo, H.M., He, Y., Sundararajan, A., Huan, L., Sangster, M.Y., 2010. Quantitative analysis of influenza virus-specific B cell memory generated by different routes of inactivated virus vaccination. Vaccine 10, 2186–2194.

Lambrecht, B.N., Kool, M., Willart, M.A., Hammad, H., 2009. Mechanism of action of clinically approved adjuvants. Curr. Opin. Immunol. 1, 23–29.

Ley, D.H., 2003. Mycoplasmosis. In: Saif, Y.M., Barnes, H.J., Glission, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E. (Eds.), Diseases of Poultry. Iowa State University Press, pp. 722–744.

Maas, R., Tacken, M., van Zoelen, D., Oei, H., 2009. Dose response effects of avian influenza (H7N7) vaccination of chickens: serology, clinical protection and reduction of virus excretion. Vaccine 27, 3592–3597.

Matsuo, K., Yoshikawa, T., Asanuma, H., Iwasaki, T., Hagiwara, Y., Chen, Z., Kadowaki, S.E., Tsujimoto, H., Kurata, T., Tamura, S.I., 2000. Induction of innate immunity by nasal influenza vaccine administered in combination with an adjuvant (cholera toxin). Vaccine 24, 2713–2722.

Nicholson, K.G., Thompson, C.I., Klap, J.M., Wood, J.M., Batham, S., Newman, R.W., Mischler, R., Zambon, M.C., Stephenson, I., 2009. Safety and immunogenicity of whole-virus, alum-adjuvanted whole-virus, virosomal, and whole-virus intradermal influenza A/H9N2 vaccine formulations. Vaccine 1, 171–178.

Rauw, F., Gardin, Y., Palya, V., Anbari, S., Gonze, M., Lemaire, S., van den Berg, T., Lambrecht, B., 2010. The positive adjuvant effect of chitosan on antigen-specific cell-mediated immunity after chickens vaccination with live Newcastle disease vaccine. Vet. Immunol. Immunopathol. 134, 249–258.

Read, R.C., Naylor, S.C., Potter, C.W., Bond, J., Jabbal-Gill, I., Fisher, A., Illum, L., Jennings, R., 2005. Effective nasal influenza vaccine delivery using chitosan. Vaccine 35, 4367–4374.

Rebel, J.M., Peeters, B., Fijten, H., Post, J., Cornelissen, J., Vervelde, L., 2011. Highly pathogenic or low pathogenic avian influenza virus subtype H7N1 infection in chicken lungs: small differences in general acute responses. Vet. Res. 1, 10–20.

Reemers, S.S., van Haarlem, D.A., Groot Koerkamp, M.J., Vervelde, L., 2009. Differential gene-expression and host-response profiles against avian influenza virus within the chicken lung due to anatomy and airflow. J. Gen. Virol. 9, 2134–2146.

Reemers, S.S., Jansen, C., Koerkamp, M.J., van Haarlem, D., van de Haar, P., Degen, W.G., van Eden, W., Vervelde, L., 2010. Reduced immune reaction prevents immunopathology after challenge with avian influenza virus: a transcriptomics analysis of adjuvanted vaccines. Vaccine 38, 6351–6360.

Takada, A., Kida, H., 1996. Protective immune response of chickens against Newcastle disease, induced by the intranasal vaccination with inactivated virus. Vet. Microbiol. 1–2, 17–25.

Saluja, V., Amorij, J.P., van Roosmalen, M.L., Leenhouts, K., Huckriede, A., Hinrichs, W.L., Frijlink, H.W., 2010. Intranasal delivery of influenza subunit vaccine formulated with GEM particles as an adjuvant. AAPS J. 2, 109–116.

Stok, W., van der Heijden, P.J., Bianchi, A.T., 1994. Conversion of orally induced suppression of the mucosal immune response to ovalbumin into stimulation by conjugating ovalbumin to cholera toxin or its B subunit. Vaccine 6, 521–526.

Sui, Z., Chen, Q., Wu, R., Zhang, H., Zheng, M., Wang, H., Chen, Z., 2010a. Cross-protection against influenza virus infection by intranasal administration of M2-based vaccine with chitosan as an adjuvant. Arch. Virol. 4, 535–544.

Sui, Z., Chen, Q., Fang, F., Zheng, M., Chen, Z., 2010b. Cross-protection against influenza virus infection by intranasal administration of M1-based vaccine with chitosan as an adjuvant. Vaccine 28, 7690–7698.

Toro, H., van Ginkel, F.W., Tang, D.C., Schemera, B., Rodning, S., Newton, J., 2010. Avian influenza vaccination in chickens and pigs with replication-competent adenovirus-free human recombinant adenovirus 5. Avian Dis. 54, 224–231.

Tseng, L.P., Chiou, C.J., Chen, C.C., Deng, M.C., Chung, T.W., Huang, Y.Y., Liu, D.Z., 2009. Effect of lipopolysaccharide on intranasal administration of liposomal Newcastle disease virus vaccine to SPF chickens. Vet. Immunol. Immunopathol. 3–4, 285–289.

Vervelde, L., Janse, E.M., Vermeulen, A.N., Jeurissen, S.H., 1998. Induction of a local and systemic immune response using cholera toxin as vehicle to deliver antigen in the lamina propria of the chicken intestine. Vet. Immunol. Immunopathol. 3, 261–272.

Worrall, E.E., Sudarisman, Priadi, A., 2009. Sialivac: an intranasal homologous inactivated split virus vaccine containing bacterial sialidase for the control of avian influenza in poultry. Vaccine 31, 4161–4168.

Yoshikawa, T., Matsuo, K., Matsuo, K., Suzuki, Y., Nomoto, A., Tamura, S., Kurata, T., Sata, T., 2004. Total viral genome copies and virus–Ig complexes after infection with influenza virus in the nasal secretions of immunized mice. J. Gen. Virol. 8, 2339–2346.

Zhu, F.C., Wang, H., Fang, H.H., Yang, J.G., Lin, X.J., Liang, X.F., Zhang, X.F., Pan, H.X., Meng, F.Y., Hu, Y.M., Liu, W.D., Li, C.G., Li, W., Zhang, X., Hu, J.M., Peng, W.B., Yang, B.P., Xi, P., Wang, H.Q., Zheng, J.S., 2009. A novel influenza A (H1N1) vaccine in various age groups. N. Engl. J. Med. 25, 2414–2423.