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## Original Article

# Virus shedding after H9N2 strain challenge of SPF Chickens immunized by an experimental Nano-Adjuvant and commercial oil emulsion avian influenza (H9N2) vaccines

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## Abstract

**Background and Aims:** Vaccination of poultry has a major impact on the prevention and control of avian influenza viruses. Nanobiotechnology techniques provide a new approach for improvement of influenza vaccine efficacy. In this study, efficacy of an inactivated nano-adjuvant vaccine developed based on an endemic H9N2 virus was evaluated in SPF chickens.

**Materials and Methods:** In each three trial 40 specific pathogen free (SPF) white Leghorn chickens were used in four groups. Chickens from treatment groups (n= 10) via subcutaneous route received a single dose of the nano-adjuvant or the oil emulsion Razi® H9N2 vaccines. Chickens in the control group C received antigen only. All the birds were challenged with H9N2 strain on day 21 post-vaccination. Cloacal and tracheal swabs were taken at 1-10 days post-challenge and viral shedding was examined using inoculation of SPF embryonated eggs.

**Results:** Both vaccinated SPF chicken groups induced complete protection against clinical signs. Viral shedding in the nano-adjuvant H9N2 vaccinated chickens was completely blocked after challenge with a homologous H9N2 virus. Statistical analysis based on the protection effects of the chickens immunized with nano-adjuvant and the Razi® H9N2 vaccine showed no significant difference, but there was a significant difference to un-vaccinated group.

**Conclusions:** The results of this study indicated that the nano-adjuvant vaccine was efficacious in protection of SPF chickens over H9N2 infection. Further field experiments are needed to demonstrate the efficacy of the vaccines under field conditions.

**Keywords:** nano-adjuvant, vaccine, Influenza virus

## Introduction

Influenza viruses belong to the Orthomyxoviridae family and are comprised of three immunological distinct types, A, B and C. Type A viruses are regarded as the most significant pathogens in terms of morbidity and mortality in mammalian and bird species [1][2]. Avian influenza A viruses are highly heterogeneous, with various pathogenicity across different species. They are divided into two groups, highly pathogenic avian influenza virus (HPAI) and low pathogenic avian influenza virus (LPAI) based on their difference in virulence for chickens [3]. In poultry, LPAI-H9N2 infection has often caused slight to moderate mortality with subclinical signs including depression, respiratory symptoms, and a decrease in egg production [4] [5]. The frequent economic

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losses in the poultry industry incurred with this subtype infection have raised serious concerns worldwide [6][7][8][9][10]. Moreover, H9N2 virus considered as a public health treat due to its potential for host-range extension, virulence enhancement, and emergence of new antigenic variants [11]. The H9N2 infected cases were recently isolated from humans with respiratory illness in China[12]. In addition, the Increase replication and circulation of H9N2 viruses in poultry may trigger the eventual emergence of HPAI variants, which was shown for H5N1 virus in outbreaks in Asia and Africa [11] .

Vaccination has been demonstrated to be one of the most effective ways for prevention and control of influenza disease and infection in chickens[13][14]. Despite the mass vaccination, infections of poultry with H9N2 subtype have been obviously widespread in domestic poultry with prevalence ranged from 28.7% to 71% due to antigenic diversity of the viruses [15][16][17]. Thus an exact controlling strategy to provide complete protection against the infection is required. In this regard, various vaccine have been developed and some of them shown efficacy in experimental studies. The most licensed influenza vaccines have been composed of whole viruses inactivated by formalin at the final concentration of 0.1% or other chemicals [18]. Although the inactivated vaccines have the advantages of a high safety profile over live attenuated vaccine, these vaccines may suffer from a relatively lower immunogenicity. Strategies for improving efficacy of the current inactivated vaccines have focused on developing a better adjuvant. Enhanced and directed immune responses to the killed vaccine can potentially be improved by modulating the vaccine formulation using nanotechnology. Aplication of this technology allows improved antigen stability and immunogenicity, targeted delivery and slow release with fewer side effects by the co-administration of adjuvant [19]. In order to elicit broader and more potent immunity than traditional influenza vaccines, we design an inactivated nano-adjuvant vaccine against a local isolate of H9N2 virus. However, infection of the LPAI viruses were generally overlooked, owing to the lack of clinical symptoms, viral shedding is considered as an important and crucial step in protecting poultry against the infection.

## Methods

**H9N2 avian influenza nano-adjuvant vaccine.** The experimental nano-adjuvant H9N2 vaccine was developed using A/Chicken/Iran/ZMT-101/98 (H9N2) reference virus strain [6] and used for immunization of specific pathogen free (SPF) chickens.

**Vaccination procedure.** Forty white Leghorn SPF chicks were divided in four groups, A to D (n=10), and reared in isolator cabinets (Bell Isolation System®) immediately after hatching. Chickens in group A were subcutaneously injected by one dose (0.5 ml) of the nano-adjuvant vaccine at 18 days of age. Group B were vaccinated with the oil emulsion Razi® H9N2 vaccine at the same manner. Control groups C were received of 0.5 ml of the H9N2 antigen subcutaneously at the same age. Group D was considered as negative control. Twenty-one days after the initial vaccination, all chickens in groups A, B, and C, and non-vaccinated control birds in group D were challenged with 107 EID50/ml of the local reference H9N2 virus, with intravenous pathogenicity index of 0.26, in a volume of 100 µl via intraocular and intranasal routs. The chickens were daily observed for 10 days post challenge and viral shedding were examined from cloaca and trachea samples. Cloacal and tracheal swab samples were taken from all birds in each group at 1-10 days post-challenge. The samples placed in transfer media containing 1ml PBS solution (pH 7.2), 10.000 IU/ml Penicillin, 1 mg/ml Streptomycin sulfate, and 100µg/ml Kanamycin and examined for influenza virus isolation. The experiment procedure was conducted on three separate trials.

**Histopathological examination.** Necropsies were performed immediately after death of challenged birds. Samples of bursa of Fabricious were collected, fixed in 10% phosphate-buffered formalin, and embedded in



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paraffin Sections of the formalin fixed samples were stained with hematoxylin and eosin.

**Virus isolation.** Virus isolation from cloaca and trachea swabs were performed according to the standard methods [20]. Briefly, a volume of 0.2 ml of each sample prepared for virus isolation was inoculated into allantoic sac of 10 day-old SPF embryonated eggs. The inoculated eggs were incubated at 37°C for up to 7 days. Eggs were candled daily and dead embryos within 24 hours after inoculation were discarded and considered as bacterial contamination. The haemaglutination (HA) and haemaglutination inhibition (HI) tests were carried out to screen for the potential presence of H9N2 virus by using 0.5 % chicken RBCs and specific H9N2 antuserum. (OIE 2008).

**Statistical analysis.** All the statistical analysis was performed using the SPSS software, version 16.0 (SPSS, Chicago, Illinois). Differences between groups were assessed using Student's t test and $P<0.05$ was considered statistically significant.

## Results

**Immunization assay.** Tracheal and cloacal swabs were collected each day from 1 to 10 post challenge for detecting virus shedding. The swab samples were used to virus isolation in SPF embryonated chicken eggs. Results of viral shedding screened by virus isolation in SPF chickens vaccinated with the nano-adjuvant vaccine compared to the commercial inactivated H9N2 vaccine and influenza antigen without adjuvant were shown in table 1. There were no obvious evidences based on virus presence in trachea and cloacal samples; after challenge in A and B groups. In the non-vaccinated C group which received the antigen without adjuvant, virus isolated from trachea at 5 and 9 days post challenge. The control birds had virus shedding from the trachea at 2, 5 and 7 days post challenge and from the cloaca at 9 day post challenge. The ratio of viral shedding in vaccinated chickens to the total number of examined chickens in each group was estimated. The ratio revealed that all vaccinated chickens in groups A and B were protected against challenge when compared to control non-vaccinated birds. No virus was recovered from the trachea and cloaca swabs collected from the vaccinated birds, while virus was detected in control chicks. A significant difference ($P<0.05$) was observed between the vaccinated and non-vaccinated groups, while no difference was recorded between the nano-adjuvanted vaccine and RAZI H9N2 vaccine.

**Necroscopy and histopathological examinations.** Gross lesions were not apparent in the examined tissues taken from vaccinated chickens. The bursa of control groups were hypertrophic and congested at 6 and 8 days post challenge (Fig. 1). In some cases fibrinonecrotic casts were observed at 6 and 8

**Table 1.** Protection against H9N2 avian influenza virus induced by nano-adjuvanted and commercial inactivated vaccines measured by reduction in number of viral shedding from tracheal and cloacal samples.

| Groups | | Virus isolation , Days postchallenge | | | |
|---|---|---|---|---|---|
| | | 2 | 5 | 7 | 9 |
| (A) Nano-adj vaccine | Tracheal swab | – | – | – | – |
| | Cloacal swabs | – | – | – | – |
| (B) Razi vaccine | Tracheal swab | – | – | – | – |
| | Cloacal swabs | – | – | – | – |
| (C) (10 7.9 EID50influenza antigen) | Tracheal swab | – | 4/10 | – | 2/10 |
| | Cloacal swabs | – | – | – | – |
| (D) (Control) | Tracheal swab | 5/10 | 8/10 | 6/10 | – |
| | Cloacal swabs | – | – | – | 2/10 |



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days post challenge in lung (Fig. 2). At 6 and 8 days post challenge, changes in the bursa consisted of lymphocyte depletion were observed (Fig. 3).

**Fig. 1.** Hypertrophy and congestion in Bursa of Fabricius prepared from dead birds challenged with ZMT-101 strain in control group at 6 and 8 days poste challenge.

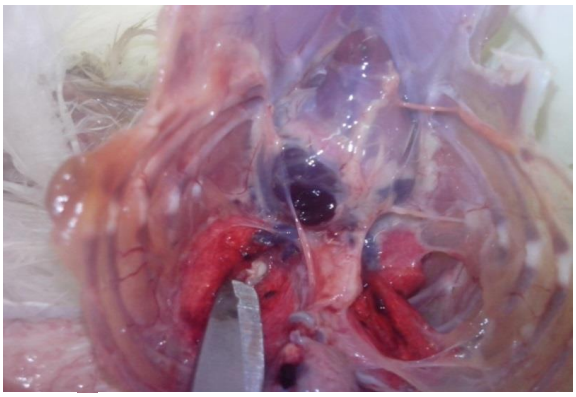

**Fig. 2.** Fibrinous casts in bronchi. Birds that died from control group challenged with ZMT-101 strain had hypertrophy and Fibrinous casts at 6 and 8 days poste challenge.

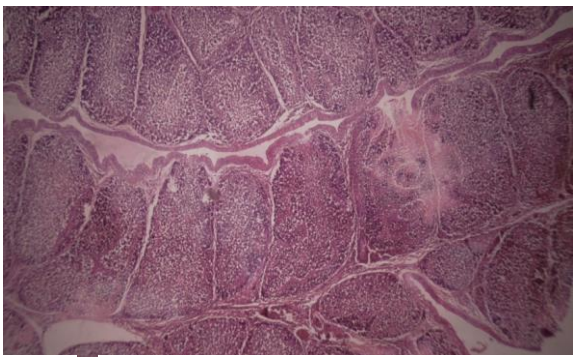

**Fig. 3.** Lymphocyte depletion in the cloacal bursa. Birds that died from control group birds challenged with ZMT-101 strain had lymphocyte depletion in the Bursa of Fabricious
at 6 and 8 days post challenge, x 40.

## Discussion

Vaccination of poultry is a recommended strategy to prevent the transmission of H9N2 avian influenza viruses in several Asian and Middle-East countries [21]. Despite the widely administration of the oil-based inactivated vaccines, improvements in antigen content, the used adjuvant, and vaccine delivery are clearly required due to concerns about the weak immunogenicity of these vaccines. Recently nano-particles as immunopotentiatores and vaccine delivery system are introduced[22]. In order to improve the immune responses against H9N2 viruses, we developed nano-adjuvanted vaccine using a local isolate encapsulated in a biodegradable agent (data not shown). Due to mild clinical signs such as respiratory signs and egg drop associated with H9N2, virus reisolation rates from tracheal and cloacal samples during infection period are measured for vaccine potency testing. In the present study, to assess the protective efficacy of the developed H9N2 inactivated vaccine, groups of the vaccinated SPF chickens were challenged with 107 EID50/ml of a homologous H9N2 virus. Tracheal and cloacal swabs were inoculated into the 9 day old SPF embryonated chicken eggs for detecting virus shedding. The results showed that no virus was detected in any swabs collected from all vaccinated chickens. Cloacal swabs taken from chickens in group C received the original antigen were negative for virus isolation; while the H9N2 viruses were isolated from the tracheal swabs at 5 and 7 days post challenge. The virus isolation and histopathology results indicated that the H9N2 virus dose not replicate in vaccinated chickens. We did not observe any clinical signs in the vaccinated chickens, so either vaccinated chickens with the experimental nano-adjuvant H9N2 vaccine or the licensed Razi vaccine were protected against the challenge when compared to control unvaccinated birds. The possible explanation for the results is specifically related to the efficacy of the developed H9N2 nano-particle vaccine.

Despite all the control measures taken, H9N2 viruses continue to circulate in poultry in Iran



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and Middle East. The co-circulation of H9N2 viruses with other respiratory viruses and mass vaccination may raise the question whether the vaccine has afforded protection from other H9 viruses[23]. To provide sufficient protection against influenza infection it is recommended that the HA serotype of the vaccine and field challenge virus should be identical. Overview on the relationships between HA sequences of these viruses revealed that the circulated viruses are not evolutionarily widely different form the vaccine strain. Also, they did not acquire mutational pressures that alter the properties of the molecule to generate a new antigenic variant[24]. Comparison the relative immunogenicity and cross-protective efficacy of H9N2 G1 viruses indicated protection in mice against subsequent challenge with the homologous or heterologous H9N2 virus[25]. All of the HA sequences of Iranian H9N2 isolates which deposited in GenBank are belong to the G1-like[24] and seems that can induce more cross-reactive antibodies against both homologue and/or heterologue viruses. Our results confirm the subject where the developed H9N2 nano-particle vaccine is able to provide 100% protective efficiency to SPF chickens. After challenge with A/Chicken/Iran/ZMT-101/98 (H9N2) virus, chickens that received oil emulsion Razi® vaccine showed no sign of disease indicating the licensed vaccine induced complete protection against the homologous challenge.

Failure of vaccination, in some cases, is related to the in used vaccine adjuvant. Combination of adjuvant and the antigen is one of the efficient ways toward induction of appropriate immune responses and improving the vaccine efficacy. Oil in water and water in oil adjuvants are widely employed in formulation of inactivated influenza vaccines [26]. Due to some limitations, within past few years, rapid developments have been made to use nanoparticles for site-specific delivery of vaccines[27]. In our main project we used a biodegradable nanoparticle as potential delivery vehicles for vaccine antigen because it is assumed that the composition will be capable of enhanced immune responses. Here, potency of the developed nano-particle H9N2 vaccine was evaluated in SPF chickens. Evidence of the virus shedding in challenge experiments showed that the vaccine is efficacious in SPF chicks, while antigen without adjuvant group provides less protective efficiency to the chickens. Induction of protective immune responses against H9N2 influenza virus in commercial flocks is targeted in the future study.

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