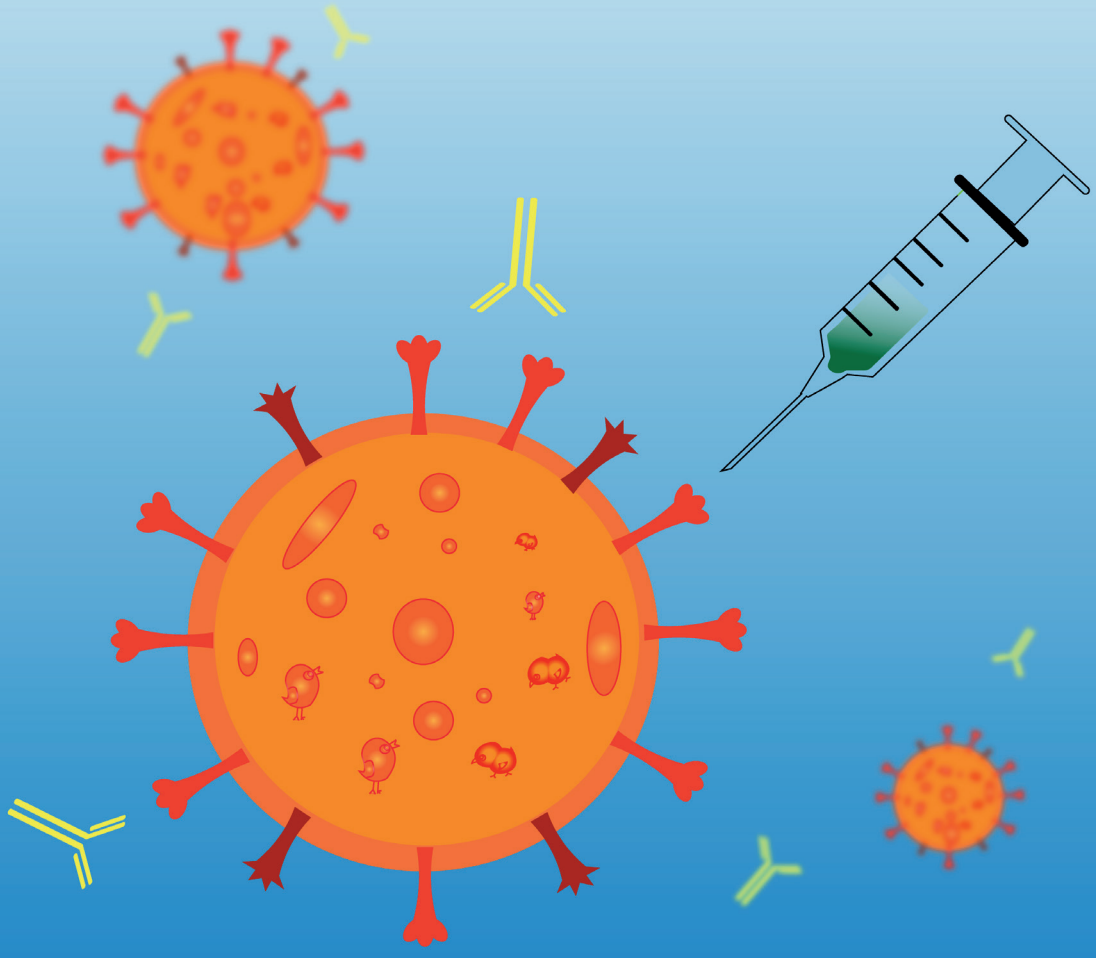
Development of immune response against H9N2 avian infuenza after vaccination



Xue Pan

Propositions

1. The recombinant turkey herpesvirus (H9) vaccine reduces transmission of H9N2 AIV in poultry.
   (this thesis)

2. Intravenous injection of chicken-interferon-α overcomes maternal antibodies interference to vaccination in chicken.
   (this thesis)

3. Scientists should pay more attention to transmission of SARS-CoV-2 rather than only individual infection.

4. Strict vegan is unhealthy for young and elderly people.

5. Social media increasingly influence scientific decisions.

6. Monkeypox is easy to control.

Propositions belonging to the thesis, entitled：

Development of immune response against H9N2 avian influenza after vaccination

Xue Pan
Wageningen, 11-1-2023

Development of immune response against H9N2 avian influenza after vaccination

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Development of immune response against H9N2 avian influenza after vaccination

Xue Pan

Thesis

Submitted in fulfilment of requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus,
Prof. Dr. A.P.J. Mol,
in the presence of the
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to be defended in public
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Development of immune response against H9N2 avian influenza after vaccination

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Abstract

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The emergence of severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) spilled over from animals to humans is a catastrophe killing more than six million people so far according to World Health Organization (WHO). Like SARS-CoV-2, the respiratory virus, H9N2 avian influenza virus (AIV), can also spill over from animals to humans, and is endemic in poultry flocks in several regions of the world. Some researchers are concerned that H9N2 AIV may be a spark to the emergence of the next influenza pandemic, either directly crossing the species barrier or through the donation of internal genes to a pandemic virus. Like dealing with SARS-CoV-2, vaccination is the main and most effective strategy to control H9N2 AIV. However, most vaccination programs against H9N2 AIV have shown to be ineffective in poultry in practice. Therefore, my project was 1) to figure out what factors may interfere with the H9N2 vaccine efficacy to sufficiently reduce transmission in poultry, 2) to come up with some ideas to improve the vaccine efficacy, and 3) to explore the mechanisms that influence the vaccine efficacy. In chapter 2, I went to several poultry farms for more than three months to collect surveillance data for H9N2 vaccination failure. The data suggests that maternal-derived antibodies (MDAs) may be one of reasons for H9N2 vaccination failure in poultry, which was further corroborated by animal experiments in broilers and specific pathogen-free (SPF) chickens in laboratory in the same chapter 2. In chapter 3, I developed a CpG ODN-based adjuvant that could help the H9N2 inactivated whole virus (IWV) vaccine overcome MDAs, triggering potent humoral immune responses and cytokinesis mRNA expression. In chapter 4, in order to stop transmission of H9N2 AIV, I used turkey herpesvirus (HVT) to express H9 HA protein. The recombinant virus (rHVT-H9) successively induced strong humoral and cellular immune responses, reducing virus shedding and transmission even in the presence of MDAs. In chapter 5, I explored some mechanisms of MDAs interference and found that only complete MDAs interfered with immune responses to vaccination instead of the antigen-binding portion ($F(ab)_2$) or the Fc-binding portion (Fc). In addition, type I chicken interferons (ch-IFNs) helped the H9N2 IWV vaccine overcome MDAs interference inducing potent humoral immune responses. Finally, in chapter 6, I discussed avian immune response after vaccination, the mechanisms of MDAs interference, new measurements of vaccine efficacy against transmission of H9N2 AIV and strategies to improve vaccine efficacy to stop transmission of H9N2 AIV in poultry. Overall, this thesis provides a deeper understanding of H9N2 vaccination failure in poultry and may spark some ideas to stop transmission of respiratory viruses.

Contents

1

General introduction

1.1 Overview of H9N2 avian influenza virus

H9N2 avian influenza virus (AIV) is the most prevalent and harmful low pathogenic avian influenza virus (LPAIV) in the world, which not only seriously harms animal husbandry but also poses a huge challenge to public health. H9N2 AIV was first isolated from turkeys in Wisconsin, USA in 1996 (Homme and Easterday 1970). In the following decades, the virus gradually spread in the Northern USA and Eurasia. H9N2 AIV was isolated from chickens in live poultry market in Hong Kong in the early 1990s and became endemic in poultry in Asia, the Middle East and North and West Africa (Shortridge 1992). H9N2 AIV has developed to be a persistent and pernicious virus in chickens and other birds in Asia such as quail, pheasant, partridge, etc. (Guan et al. 1999, Liu et al. 2003, Guo et al. 2000). Currently, H9N2 AIV is widespread around the world **(Fig. 1)**.

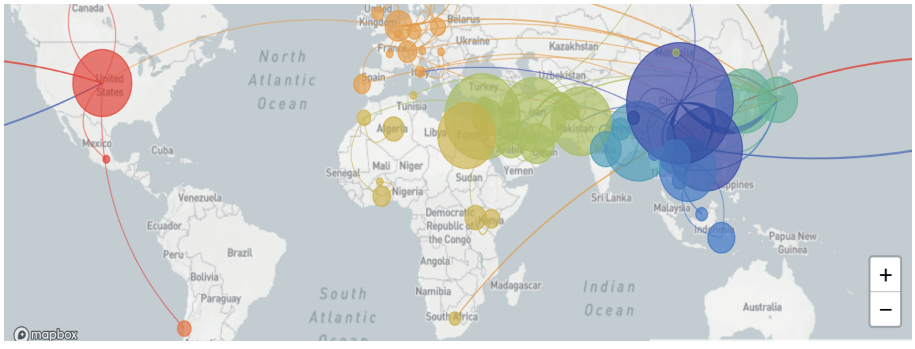

Figure 1. Transmission of H9N2 avian influenza virus in the world. There were 1384 HA genomes sampled between July 1976 and January 2022 in 56 countries. (https://nextstrain.org/flu/avian/h9n2/ha)

Although belongs to LPAIV, the damage caused by H9N2 AIV should never be underestimated. On the one hand, H9N2 AIV can provide inner genes for highly pathogenic avian influenza virus (HPAIV) such as H5N1 and H7N9, infecting 863 people with 455 deaths and 1568 people with 616 deaths respectively since early 2013 according to WHO. In addition, H9N2 AIV contributes inner segments to other novel zoonotic AIV viruses such as H5N6, H10N8 and H10N3 which also have been shown to infect humans (Wang et al. 2021, RahimiRad et al. 2016, Shen et al. 2016). On the other hand, mixed infections of H9N2 AIV with other pathogens, such as infectious bronchitis virus (IBV), *Mycoplasma gallisepticum*, *Staphylococcus aureus*, *Escherichia coli*, *Avibacterium paragallinarum*, *Ornithobacterium rhinotracheale* and/or immune suppressive agent will enhance the severity of the clinical syndrome and higher rates of mortality in poultry, extending the period of H9N2 AIV shedding (Sun and Liu 2015, RahimiRad et al. 2016, Dadras, Nazifi and Shakibainia 2014,

Umar et al. 2021, Hassan et al. 2017, Mosleh et al. 2017, Kishida et al. 2004, Nili and Asasi 2003). Furthermore, H9N2 AIV itself can directly infect humans and other animals in nature including wild birds, pigs, dogs, cats, minks and horses (Zhou et al. 2014, Guan et al. 2000, Okamatsu et al. 2008, Shanmuganatham et al. 2014, Peacock et al. 2016, He 2012). An increasing number of humans have been infected by H9N2 AIV since 2015 and especially in the last three years according to WHO **(Fig. 2)**. Several H9N2 virus strains have evolved human receptor-binding specificities, pH-stable HA proteins and internal gene cassettes (e.g., genotype 57), which allow more efficient replication and transmission in humans (Thuy et al. 2016, Zhu et al. 2018, Wang et al. 2019, Han et al. 2019). Therefore, some researchers are concerned that H9N2 AIV may be a spark to the emergence of the next influenza pandemic (Caceres et al. 2021, RahimiRad et al. 2016, Peacock et al. 2019).

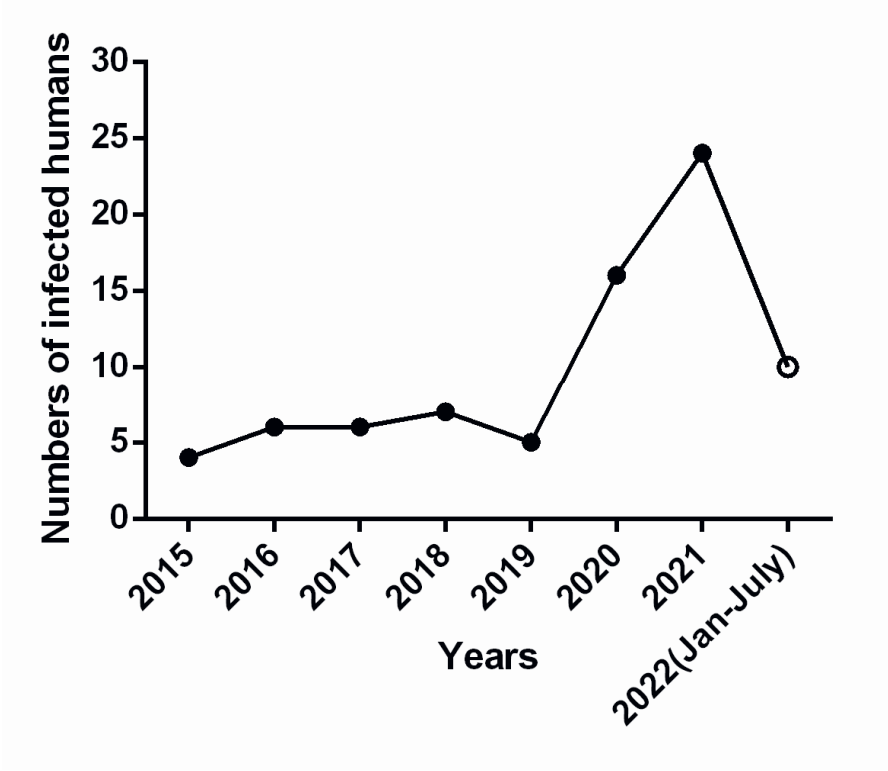

Figure 2. The number of infected humans by H9N2 AIV in the world every year. (https://apps.who.int/iris/handle/10665/351652)

1.2 Vaccination against H9N2 AIV in poultry

Because of the huge damage caused by H9N2 AIV, people have been using some strategies to control H9N2 AIV in poultry. To eradicate AIV, two approaches are being applied in different countries in the world: stamping out and vaccination. Stamping out, culling of infected birds and the flock of birds with suspected exposure to the virus, has been used as the primary control measure in those countries where disease has recently been introduced (Suarez 2012, Suarez 2005). However, stamping out is mainly aimed at HPAIV such as H5 and H7, but it is considered ineffective (or too expensive) for controlling H9N2 AIV which has already been in prolonged circulation in poultry. In addition, stamping out comes at a high economic and social cost. On the other hand, vaccination is the main and most effective strategy to control H9N2 AIV. The H9N2 inactivated whole virus (IWV) vaccines have been widely used in poultry farms. By far, many countries including China, South Korea, Israel, Pakistan, Egypt, Morocco, Iran and United Arab Emirates (UAE) are using vaccines as a key approach for preventing H9N2 disease in poultry (Banet-Noach et al. 2007, Naeem and Siddique 2006, Kilany et al. 2016, Lau et al. 2016, Zhang et al. 2008, Bahari et al. 2015).

1.3 Vaccination failure in practice

Although vaccines have been used against H9N2 AIV in poultry for many years, H9N2 vaccination failure occurs regularly. As a result, the virus is still circulating and prevalent in many regions around the world. In experimental studies, most H9N2 vaccines work well in specific pathogen-free (SPF) chickens in laboratory stimulating potent immune responses, but it is not the case when applying those vaccines in poultry. Most vaccination programs against H9N2 AIV in poultry have shown to be ineffective (Gu et al. 2017, Peacock et al. 2019, Bahari et al. 2015). Those vaccines may reduce some clinical signs and provide some clinical protection, but they work poorly in stopping transmission of H9N2 AIV in chickens (Cui et al. 2021). Therefore, it is common in vaccinated poultry farms that there are few clinical signs, but the virus transmission still occurs, which is called “silent spread”. This kind of spread of H9N2 AIV among poultry can work as a potential driving force in antigenic drift and the selection of immune escape mutants (Pu et al. 2015, Park et al. 2011, Davidson et al. 2013, Peacock et al. 2017, Bahari et al. 2015, Zhang et al. 2008). Furthermore, the long-term spread of H9N2 AIV increases the possibility to cross the species barrier to infect other animals including humans, and thus theoretically increases the zoonotic potential and pathogenicity (Sealy et al. 2019). Therefore, it is necessary to figure out why H9N2 vaccination failure occurs in poultry in practice and how to overcome this problem.

1.4 Possible reasons for H9N2 vaccination failure

Antigenic distance between vaccine and field strain is considered to be the most important factor in AIV vaccine failure in poultry. Thus, updating vaccine constantly seems to be indispensable (similar to vaccination against human influenza). As a consequence, it would be very troublesome and cost lots of money (Domenech et al. 2009, Kapczynski and Swayne 2009, Capua and Cattoli 2013, Balish et al. 2010, Giovanni et al. 2011, Marisa et al. 2009). The hemagglutination inhibition (HI) assay has been regarded as a good way to measure the changes in antigenicity and has been used to assess the antigenic properties of AIV for years (Donald and Isaacs 1954). Antigenic distance between vaccine and field strains can be calculated by the discrepancies in HI titer between the field virus and the vaccine virus tested with the serum against the vaccine virus. The bigger the discrepancies are, the larger the antigenic distance will be. For instance, when the HI titers of the vaccine strain are 2^{10} and of the field strain is 2^3, the antigenic distance between vaccine and field strain is 2^7 in this case. However, if the titers of the vaccine virus are already 2^2 or 2^1, it does not matter that the titers against the field virus are less or not (Sitaras et al. 2016a, Sitaras et al. 2016b). In addition, when antigenic difference is maximally 2^5-2^6, it implies that with a titer after vaccination of 2^{10} - 2^{11} antigenic differences do not matter. They would only reduce the titer to 2^4 - 2^6 which would still be more than enough to protect against transmission of H5N1 AIV. High immune responses and a proper vaccine coverage would compensate for the antigenic distance and protect animals from infection and transmission (Senne and Suarez 2004, Muhammad Athar et al. 2011, Swayne, Chang-Won and Erica 2006, Calogero et al. 2010, Jennifer et al. 2010, Tian et al. 2005, Swayne et al. 2015, Sitaras et al. 2016b). Field studies have confirmed that compared to antigenic distance, high HI titers and vaccine coverage play a more vital role in preventing transmission of AIV. When there are higher HI titers and vaccine coverage among poultry, the possibility of transmission and infection by AIV will become lower (Magalhães, Pfeiffer and Otte 2010, Ellis et al. 2004).

Mixed infection and/or concurrent infection of H9N2 AIV with other pathogens or live vaccine may contribute to the vaccine failure in stopping transmission of AIV. Mixed infections of H9N2 AIV with other respiratory pathogens mentioned above, can exacerbate H9N2 AIV infection, replication or dissemination, extending the period of H9N2 AIV shedding and resulting in severe clinical disease and variable mortality (Dadras et al. 2014, Sajid et al. 2015, Hassan et al. 2017, Mosleh et al. 2017, Perk et al. 2004, Kishida et al. 2004, Nili and Asasi 2003). Therefore, the mix infection in poultry may reduce the H9N2 vaccine efficacy. Furthermore, co-infection with immunosuppression diseases like Marek's disease virus (MDV), reticuloendotheliosis

virus (REV), infections bursal disease virus (IBDV), chicken infectious anemia (CIAV) in chicken will increase illness severity and also reduce vaccine efficacy (Sun et al. 2017, Schat and Skinner 2014, Spackman, Stephens and Pantin-Jackwood 2018). In addition, other factors, such as improper vaccination procedure, incorrect use of vaccine, poultry density, condition of chicken etc, may also be the reasons for H9N2 vaccination failure in vaccinated poultry.

1.5 Interference by maternal-derived antibodies

Maternal-derived antibodies (MDAs) are also a possible reason for H9N2 vaccination failure in poultry. Previous studies demonstrated that MDAs are critical for newborn chickens at the beginning of their lives when they are vulnerable protecting them from pathogen infections (Maas et al. 2011, Forrest et al. 2013). However, MDAs may also inhibit active immune responses at the same time (Poetri et al. 2011, Maas et al. 2011, Forrest et al. 2013). The transfer of maternal immunity through the yolk sac can give rise to the commercial H5N1 vaccine failure in Egyptian poultry (Kim et al. 2010). Other papers have also reported the negative effect of MDAs on inactivated AIV and vector vaccines immunogenicity and efficacy in chickens (Bublot et al. 2010, Maas et al. 2011, David et al. 2010, Richard-Mazet et al. 2014). Applying different vaccines in mothers and their offspring is a good way to tackle MDAs interference problem, but this may only work for those viruses that carry at least two different proteins which have multiple neutralizing epitopes, such as Newcastle disease virus (NDV) (Gharaibeh, Mahmoud and Alnatour 2008, Sachin et al. 2011, Karaca et al. 1998). Obviously, this strategy is not available for H9N2 AIV since most of the multiple neutralizing epitopes among different H9N2 AIV with the same HA type are similar, which suggests that the antibodies passively transferred from mothers will be also able to combine the common epitopes in progeny vaccine, leading to the failure of vaccination (Pitcovski et al. 2017). Based on surveillance data in poultry farms, I hypothesized that H9N2-specific MDAs may interfere with immune responses after inoculating with the H9N2 IWV vaccine. Since different vaccines in mothers and their offspring is not applicable against H9N2 AIV, therefore it is important to study and develop new vaccines to overcome MDAs interference in poultry.

1.6 Overcoming maternal-derived antibodies interference

1.6.1 Inactivated vaccine

A good adjuvant may help inactivated vaccines tackle the MDAs interference problem. Traditional inflammatory adjuvants can improve adult immune response to vaccines but are less successful in neonates due to MDAs interference. Cytosine linked to a guanine by a phosphate bond containing oligodeoxynucleotides (CpG ODN) has

shown great potential as a vaccine adjuvant candidate to overcome MDAs interference in mammals. When toll-like receptor 3 (TLR-3) agonists (poly IC) and toll-like receptor 9 (TLR-9) agonists (CpG ODN) are used together as the adjuvant of inactivated measles vaccines, high titers of measles virus (MeV)-specific antibodies (both neutralizing and non-neutralizing antibodies) can be generated even in presence of MeV-specific MDAs in cotton rats after vaccination (Kim and Niewiesk 2013). In addition, Polewicz et al (Polewicz et al. 2013) found that when co-formulating pertussis toxoid (PTd) and filamentous hemagglutinin (FHA) with CpG ODN, cationic innate defense regulator (IDR) peptide and polyphosphazene (PP) into microparticle as adjuvant of inactivated vaccines against pertussis, the vaccination in the presence of MDAs offers protection against challenge infection in mice. In the presence study, I explored different types of CpG ODN and their combination as adjuvant for the H9N2 inactivated vaccine to overcome MDAs interference.

1.6.2 Viral vector vaccine

Turkey herpesvirus (HVT) is one of the potent vectors for polyvalent live vaccines and has been minimally or not at all impacted by MDAs in chickens. The cell-associated nature, the nature of the replication of the HVT vector and the lack of expression of target antigens on the surface of infected cells or by the recombinant HVT vaccine probably all contribute to avoiding the MDAs interference against vector and/or target pathogens (Bublot et al. 2007, Faulkner et al. 2013, Bertran et al. 2018, Le Gros et al. 2009). HVT-based vaccines can avoid MDAs interference against infectious bursal disease virus (IBDV) (Bublot et al. 2007, Sedeik et al. 2019, Le Gros et al. 2009), Newcastle disease virus (NDV) (Tatar-Kis et al. 2020), and H5N1 avian influenza virus to some extent (Kilany et al. 2015). Therefore, in the present study, I used HVT as a vaccine vector to express H9N2 HA protein to overcome MDAs interference.

1.7 Mechanisms of MDAs interference

Understanding the underlying mechanisms of MDAs interference will contribute to overcoming the problem. However, the mechanisms of MDAs interference with immune response in birds have not been extensively explored. Some researchers have explored the mechanisms in mammals, but the conclusion is still debated. In mammal, there are mainly five hypotheses about the mechanisms of MDAs interference: 1) antigen neutralization (Albrecht et al. 1977): MDAs neutralize and damage the replication of live virus vaccine through blocking attachment and entry of virus into cells; 2) epitope masking (Bergstrom, Xu and Heyman 2017, Brüggemann and Rajewsky 1982, Heyman and Wigzell 1984): immunodominant epitopes in the virus are recognized and bound by MDAs, blocking the recognition by B cell receptor

(BCR); 3) inhibition by Fcγ receptor IIB (FcγRIIB)-mediated signaling (Kim et al. 2011, Edwards 2015): BCR, antigens, MDAs and Fc receptor (FcγRIIB) combine together to downregulate the signal of B cell stimulation; 4) clearance of MDA-coated vaccine antigens (Siegrist 2003): MDAs bind to viral antigen on the cells, which normally would attract phagocytes and natural killer (NK) cells to clear the virus-infected cells; 5) shaping the early-life B cells repertoire (Vono et al. 2019): MDAs interfere with antigen-specific plasma cells and memory B cells by shaping the early-life B cells repertoire within germinal centers. In birds, however, little research has been done to study MDAs interference.

1.8 Transmission and mathematical modeling

When assessing the efficacy of vaccines in the control of infectious diseases, the ability to control the spread of viruses at the population level should be more important than only clinical protection and individual infection. Typically, most vaccine studies against influenza viruses mainly focus on protection from clinical signs and individual infection, while the transmission of viruses is not taken into account. The consequences of prolonged circulation of viruses (new variants, infection of other species including humans) may be more harmful than clinical disease. Hence, it is essential to study the vaccine efficacy in stopping transmission of H9N2 virus in poultry.

Transmission of viruses can be assessed by the reproduction ratio (R) which is the average number of secondary infections caused by a one typical infected animal (Diekmann, Heesterbeek and Metz 1990). Mathematical modeling is used to evaluate the R value. Over the last few decades, mathematical modeling has been extensively used in many subjects, such as agriculture, aerospace, physics, etc. Especially during the COVID-19 pandemic caused by SARS-CoV-2, a growing number of people realized the importance of mathematical modeling, and an increasing number of mathematical models have been built for estimating the R value of SARS-CoV-2, helping make strategies against the virus. To eradicate viruses, we always aim for a $R < 1$, which indicates that a typical infectious individual will infect on average less than one susceptible individual and the pathogen will disappear from the host population in the end.

1.9 Project aim and outline of the thesis

I hypothesized that H9N2-specific MDAs may interfere with immune responses after inoculating with the H9N2 IWV vaccine in poultry. Therefore, my project aimed to identify whether MDAs interfere with immune responses and developed some new vaccines to overcome MDAs interference with the ultimate goal to stop transmission

of H9N2 AIV in poultry. However, studying MDAs-related research is difficult because the final MDAs transferred from dams have a high degree of variability in individual broilers (Gharaibeh, Mahmoud and Al-Natour 2008). Hyperimmune serum which contains mostly IgY has similar isotype proportions to MDAs and therefore passively transferred antibodies (PTAs) can be used to mimic MDAs in SPF chickens (Hamal et al. 2006, Faulkner et al. 2013).

In chapter 2, I went to several poultry farms in China and did an epidemiological investigation for more than three months. Based on surveillance data in poultry farms, I hypothesized that H9N2-specific MDAs may interfere with immune responses after inoculating with the H9N2 IWV vaccine. Animal experiments in broilers in poultry farms and in SPF chickens using PTAs to mimic MDAs in laboratory supported my hypothesis. Therefore, **in chapter 3**, I developed a new CpG ODN-based adjuvant for the H9N2 IWV vaccine to overcome MDAs interference. I first selected which types of CpG ODN could stimulate the best immunomodulatory activities in chicken cell line *in vitro*. Next, I performed two animal experiments to identify the best adjuvant candidate for the H9N2 IWV vaccine and the minimal dose of the adjuvant in the presence of PTAs in chickens. Furthermore, **in chapter 4**, I used HVT as a vaccine vector to express H9 hemagglutinin (HA) protein. PCR analysis, western blot, indirect immunofluorescence assay (IFA) and plaque assays were used to identify the characters of the recombinant viral vector vaccine (rHVT-H9) *in vitro*. Later, I designed a transmission experiment in chickens that received PTAs to assess the vaccine efficacy in stopping transmission of H9N2 AIV, and used mathematical modeling to calculate R value. **In chapter 5**, I explored the mechanisms of MDAs interference. Antibodies from different species against different antigens were passively transferred into SPF chickens to mimic complete H9N2-specific MDAs ($F(ab)_2$ + Fc), the antigen-binding portion of H9N2-specific MDAs ($F(ab)_2$) and the Fc-binding portion of H9N2-specific MDAs (Fc). Using this model, I explored which portion of MDAs interfered with H9N2 vaccination in chickens. In addition, chicken interferons (Ch-IFNs) were used to overcome MDAs interference. Finally, in the last chapter, **chapter 6**, I discussed immune responses after vaccination, some new measurements to assess the efficacy of vaccines in stopping transmission of H9N2 AIV and future perspectives of vaccines in poultry based on new measurements. A schematic outline of the thesis with topic per chapter is shown in **Figure 1.3**.

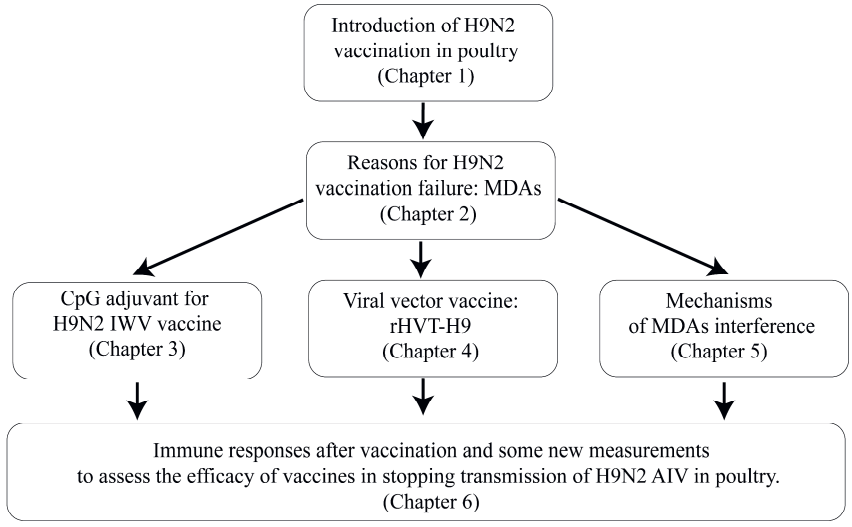

Figure 1.3 Outline of the thesis with topic per chapter.

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2

Maternal-derived antibodies hinder antibody response against H9N2 AIV inactivated vaccine in poultry

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Abstract

The H9N2 subtype avian influenza virus (AIV) inactivated whole virus (IWV) vaccine has been used extensively in poultry farms, however, it often fails to stimulate potent immune responses in poultry although it works well in laboratory experiments. Based on surveillance data in poultry farms, we hypothesized that maternal-derived antibodies (MDAs), the transfer of maternal antibodies through the yolk sac, are possible reasons for vaccination failure in poultry. An animal experiment in a poultry farm showed that 1-day-old broilers with MDAs were not triggered potent humoral immune responses by the commercial H9N2 IWV vaccine. Using passively transferred antibodies (PTAs) to mimic the presence of MDAs in specific pathogen-free (SPF) chickens in laboratory, the same results were observed that the H9N2-specific PTAs inhibited humoral immune responses against the H9N2 IWV vaccine. After challenge with homologous H9N2 virus, the virus was still detected in oropharyngeal swabs of the vaccinated chickens with PTAs, but not in the vaccinated chickens without PTAs. These results firmly supported our hypothesis. Furthermore, we explored different titers of PTAs on the efficacy of the H9N2 IWV vaccine. When different titers of PTAs were used to mimic MDAs in SPF chickens, the results showed that high (HI = 12 $\log_2$) and medium (HI = log 9 $\log_2$) titers of PTAs could reduce the generation of H9N2-specific antibodies after the first vaccination. However, a booster dose would induce a high and faster humoral immune response even under the condition of PTAs interference. Overall, our study strongly suggests that MDAs are one of the reasons for H9N2 vaccination failure in poultry.

Keywords: Maternal-derived antibodies (MDAs), Passively transferred antibodies (PTAs), Humoral immune response, Vaccination failure, H9N2 avian influenza virus (AIV)

2.1 Introduction

H9N2 subtype avian influenza virus (AIV) is the most widespread and harmful lowly pathogenic avian influenza virus (LPAIV) in large parts of the world, and therefore poses an enormous threat to both global poultry industry and human food security. In addition, H9N2 AIV might contribute to the influenza viruses that cause diseases in humans through either the donation of internal genes to highly pathogenic avian influenza viruses (HPAIVs) such as H5 and H7 AIVs or regular spillover from birds to humans (Trock, Burke and Cox 2015, Peacock et al. 2019, Pusch and Suarez 2018, Song and Qin 2020). Therefore, it is essential to find some approaches to control H9N2 AIV in poultry.

Vaccination is the main strategy for controlling H9N2 AIV in poultry. The H9N2 inactivated whole virus (IWV) vaccine is currently most used in many countries such as China, Israel, South Korea, Morocco, Egypt, Pakistan, Iran, etc. (Zhang et al. 2008, Banet-Noach et al. 2007, Naeem and Siddique 2006, Kilany et al. 2016, Bahari et al. 2015, Lau et al. 2016, Lee and Song 2013). Based on surveillance data in several poultry farms in China, in order to protect commercial broilers against H9N2 AIV, broilers are normally vaccinated three times using the H9N2 IWV vaccine before being sold when they are around forty-five-day-old: one-day-old with 0.1 ml, fourteen-day-old with 0.2 ml and twenty-one-day-old with 0.3 ml. However, most vaccination programs against H9N2 AIV have been proved to be ineffective in protection against infection or transmission in poultry although those vaccines have been claimed to work well in laboratory (Cui et al. 2021, Bahari et al. 2015, Gu et al. 2017, Peacock et al. 2019).

Unfortunately, it is still unclear what factors contribute to H9N2 vaccination failure in poultry. Antigenic distance between vaccine and endemic strain in poultry is considered to be an important factor in H9N2 vaccination failure (Kapczynski and Swayne 2009, Capua and Cattoli 2013, Balish et al. 2010, Cattoli et al. 2011). Antigenic distance can be evaluated by the differences in hemagglutination inhibition (HI) titers of for example the field virus against the field virus and of the same virus against the vaccine virus. The HI assay has been regarded as the most effective method to measure the changes in antigenicity and has been used to assess the antigenic properties of AIVs for years (Donald and Isaacs 1954). The bigger the discrepancies are, the larger the antigenic distance will be. However, Sitaras et al (Sitaras et al. 2016a, Sitaras et al. 2016b) proved that when there are enough HI titers (over 2^3) against challenge strain in over 86.5% population of vaccinated animals, the transmission of HPAIV AIV H5N1 can be stopped regardless of the antigenic distance. High immune responses and a proper vaccination coverage can compensate for the

antigenic distance and protect animals from infection and transmission (Swayne, Chang-Won and Erica 2006, Tian et al. 2005, Swayne et al. 2015, Sitaras et al. 2016a, Terregino et al. 2010, Pfeiffer et al. 2010, Abbas et al. 2011). Therefore, to get high immune responses, it is vital to figure out what factors interface with immune responses in poultry.

Maternal-derived antibodies (MDAs) are reported to interfere with immune responses in many species and hinder the efficacy of most kinds of vaccines, such as inactivated vaccine, vector vaccine, subunit vaccine and live attenuated vaccine in mammals (Niewiesk 2014, Bahgat et al. 2009, Faulkner et al. 2013, Maas et al. 2011). However, so far, no study has been performed to explore whether MDAs interfere with the efficacy of the H9N2 IWV vaccine. MDAs-related research is difficult because the final MDAs transferred from dams have a high degree of variability in individual broilers (Gharaibeh, Mahmoud and Al-Natour 2008). However, hyperimmune serum contains mostly IgY which has similar isotype proportions to MDAs and therefore can be used to mimic MDAs in specific pathogen-free (SPF) chickens (Hamal et al. 2006, Forrest et al. 2013). Therefore, the goal of the present study was to explore whether MDAs interfere with immune responses to H9N2 vaccination by using the PTAs model in SPF chickens.

2.2 Results

2.2.1 MDAs interfere with broilers' humoral immune response in poultry

The commercial H9N2 IWV vaccine has been used in poultry for more than twenty-five years in China (Jiang et al. 2012, Liu et al. 2020), and it has been proved to be very good efficacy in SPF chickens in laboratory, nevertheless the H9N2 AIV is still prevalent among poultry farms in the field. To explore what factors may contribute to the commercial H9N2 IWV vaccine failure in the field, the same age SPF chickens and commercial broilers were raised together and vaccinated to show their immune response. The results were shown in **Figure 1**. The HI titers of one-day-old commercial broilers were 9.6 ± 0.5 $\log_2$ at the moment of vaccination which we interpreted to be because of MDAs and the titers went down gradually to approximately 3 $\log_2$ at day 28 when not vaccinated. After applying the commercial H9N2 IWV vaccine to immunize the commercial one-day-old broilers, the HI titers went down gradually, as well, not different from the PBS inoculated one-day-old commercial broilers **(Fig. 1A)**. In contrast, the HI titers of one-day-old SPF chickens were zero at day of vaccination (interpreted as no MDAs) and were significantly increased after seven days vaccination when inoculated with the commercial vaccine **(Fig. 1B)**. The HI titers of the commercial broilers after twenty-one days were low (3.6 ± 0.7 $\log_2$), interpreted as reduction in MDAs. On the other hand, the HI titers of

both commercial broilers and SPF chickens increased after vaccination when starting with twenty-one-day-old chickens (i.e. with less remaining MDAs). The HI titers of all vaccinated groups in twenty-one-day-old showed significantly higher than the corresponding PBS inoculated groups **(Fig. 1C-D)**. All the results indicated that the high titers of MDAs in broilers at the moments of vaccination are one of the reasons for H9N2 IWV vaccine failure in poultry.

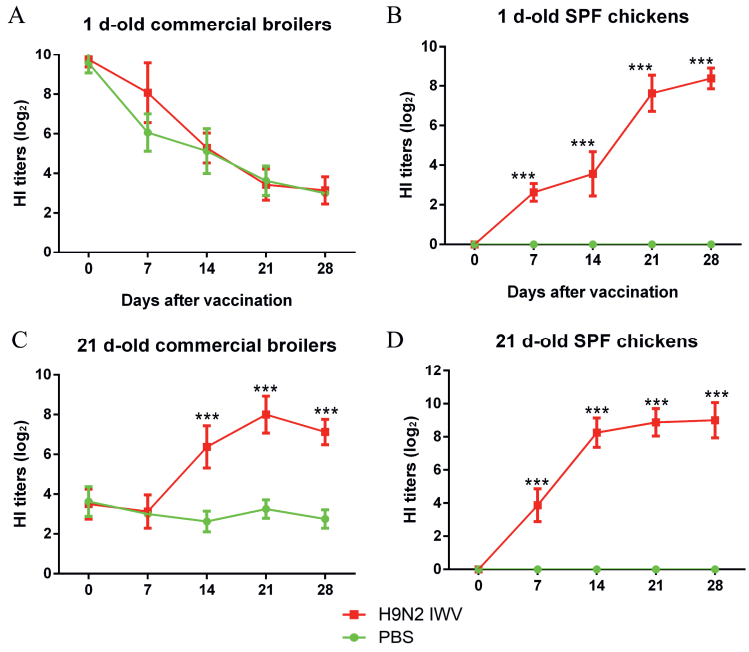

Figure 1. Seroconversion to the commercial H9N2 IWV vaccine in commercial broilers and SPF chickens. Each 1-day-old broiler (n=8) (A) and 1-day-old SPF chickens (n=8) (B) in group one was inoculated subcutaneously in the neck with 0.1 ml of commercial H9N2 IWV vaccine. Each 21-day-old broiler (n=8) (C) and 21-day-old SPF chickens (n=8) (D) in group two were inoculated subcutaneously in the neck with 0.3 ml of commercial H9N2 IWV vaccine. The same amount and age of SPF chickens and broilers in group three were vaccinated subcutaneously in the neck with 0.1 ml of PBS served as negative control. Serum of H9N2-specific antibody responses was measured weekly using the HI assay. Symbol (*) denotes differences between two groups at the same time point (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

2.2.2 The mimic MDAs by PTAs interfere with SPF chickens' humoral immune response in laboratory

To identify whether MDAs are indeed one of the reasons for the H9N2 IWV vaccine failure in poultry, PTAs were used to mimic MDAs in laboratory. One-day-old SPF chickens were passively transferred with 0.3 ml of high titers (HI = 12 $\log_2$) of H9N2-specific antibody and then vaccinated to show their immune response. The results **(Fig. 2A)** showed that after passively transferring, the HI titers of those chickens would be around 8 $\log_2$, which were similar to the HI level of one-day-old commercial broilers in the field. The dynamics of HI titers of PTAs in SPF chickens were also similar to that of MDAs of commercial broilers from 8.4 ± 0.5 $\log_2$ to 2.8 ± 0.8 $\log_2$. Two different hyperimmune sera were used to mimic MDAs in this study: one was collected from commercial hens in the field which were vaccinated with multiple kinds of vaccines including H9N2, H5N1 and H7N9 inactivated vaccines, live attenuated Newcastle disease virus (NDV) vaccine, VAXXITEK (herpesvirus of turkeys + infectious bursal disease) vector vaccine, etc; another was collected from SPF chickens in the laboratory, which contained antibodies against only H9N2 AIV. The results showed that both types of PTAs hindered the generation of H9N2-specific antibodies after vaccination. In contrast, the HI titers of those SPF chickens without PTAs went up gradually after vaccination. The HI titers of SPF chickens with PTAs showed significantly lower than those of SPF chickens without PTAs after fourteen days of vaccination with the H9N2 IW V vaccine.

2.2.3 Viral shedding and antibody titers after H9N2 challenge

MDAs interfered with humoral immune response to H9N2 vaccination in poultry. To gain insight into the MDAs interference, chickens were challenged after four weeks of vaccination and viral shedding was detected. As shown in **Figure 2B**, in oropharyngeal, viral titers of the unvaccinated group were the highest at 4.9 ± 0.4 $\log_2$ at 3 days dpc, and the viral titers were 1.5 ± 0.9 and 1.2 ± 0.7 $\log_2$ in chickens with PTAs from commercial hens and SPF chickens respectively. On the other hand, there was no viral shedding in the vaccinated group without PTAs. At 5 dpc, virus could be detected only in unvaccinated group (EID50 = 3.1 ± 0.4 $\log_2$/ 0.1 ml) in oropharyngeal samples. Virus was only detected in oropharyngeal samples but not in cloacal swabs.

Compared to unvaccinated chickens, vaccinated chickens were more likely to a high and faster antibody response after challenge. The antibody titers of vaccinated chickens with PTAs from commercial hens were from 4.6 ± 1.1 to 11 ± 0.7 $\log_2$; the antibody titers of vaccinated chickens with PTAs from SPF chickens were from 4.6 ± 0.9 to 12 ± 1.2 $\log_2$ one week later after challenge **(Fig. 2C)**.

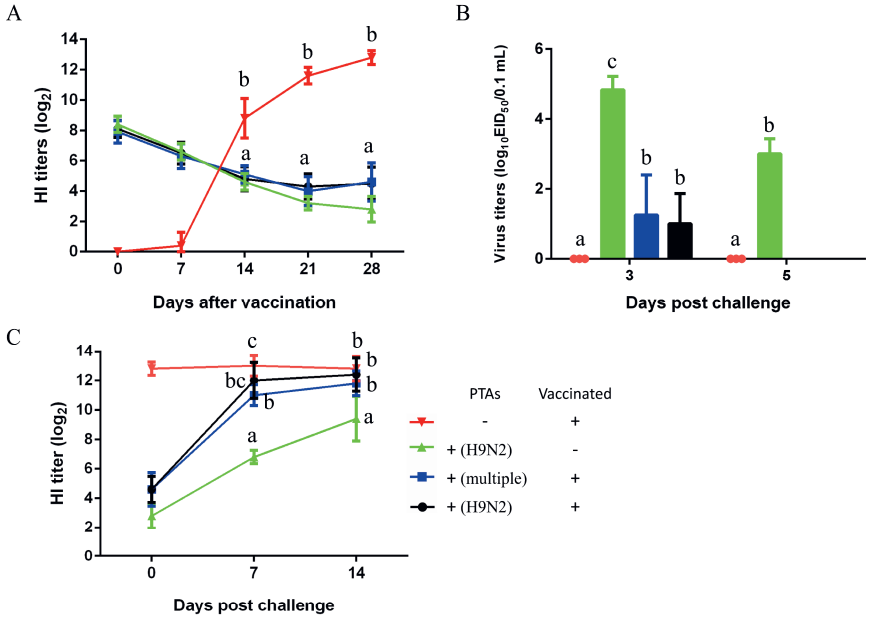

Figure 2. Mimicking MDAs by PTAs in SPF chickens and the response to vaccination and challenge. Hyperimmune serum (0.3 ml/chicken) with HI titers of 12 $\log_2$ against H9N2 virus was intravenously transferred into one-day-old SPF chickens (n = 5/group) to mimic MDAs. On the same day, chickens were vaccinated subcutaneously in the neck with 0.1 ml of the H9N2 IWV vaccine and challenged intravenously with 0.1 ml of H514 (10^6 EID_{50}/0.1 ml) at twenty-eight dpv. Seroconversion after vaccination (A), viral titers in oropharyngeal swabs after challenge (B) and seroconversion after challenge (C) were shown. Superscripts a-c denote differences among each group at the same time point.

2.2.4 High and medium titers of PTAs interfered with chickens' humoral immune response after the first vaccination but not a booster dose

To gain further understanding of the degree of interference by MDAs, high (HI=12 $\log_2$), medium (HI = 9 $\log_2$) and low (HI = 6 $\log_2$) titers of H9-specific antibodies and PBS were used as PTAs in the present study. The results showed that high **(Fig. 3A)** and medium **(Fig. 3B)** titers of PTAs indeed curbed the development of antibodies after prime vaccination, while low **(Fig. 3C)** titers of PTAs and PBS did not. After the booster vaccination, the antibodies of chickens with both high and medium titers of PTAs increased immediately from 5.6 ± 0.5 to 10.2 ± 0.4 $\log_2$ and 3.6 ± 0.5 to 10.8 ± 0.4 $\log_2$ one week later respectively, which were similar to the antibody levels of vaccinated SPF chickens without PTAs.

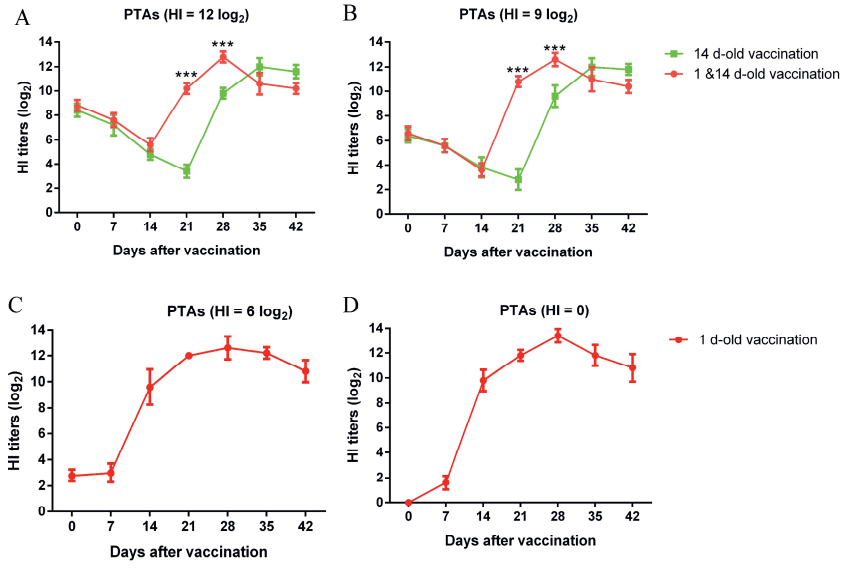

Figure 3. Seroconversion to prime or booster vaccination in SPF chickens with high, medium or low titers of PTAs. One-day-old SPF chickens were passively transferred with 0.3 ml of high (HI = 12 $\log_2$) (A), medium (HI = 9 $\log_2$) (B), low (HI = 6 $\log_2$) (C) titers of antibodies or PBS (D) per chicken intravenously (n = 10/group). Half of one-day-old chickens in each group were vaccinated subcutaneously in the neck with 0.1 ml of the H9N2 IWV vaccine. All of them were boosted with 0.2 ml of the H9N2 IWV vaccine at fourteen dpv. Serum of H9N2-specific antibody responses was measured weekly by the HI assay. Symbol (*) denotes differences between two groups at the same time point (* $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$).

2.3 Discussion

H9N2 AIV is causing enormous damage to economies and public health. To control H9N2 AIV, the H9N2 IWV vaccine has been used regularly in poultry in several countries. However, the vaccine fails to stop infection or transmission of H9N2 AIV. The study conducted in broilers and SPF chickens showed that H9N2-specific MDAs interfered with immune responses after vaccination with the H9N2 IWV vaccine in chickens. Furthermore, using PTAs model to mimic MDAs, we found that vaccinated chickens that received PTAs still shed virus through oropharyngeal route, but not in vaccinated chickens without PTAs three days after challenge. In addition, high (HI = 12 $\log_2$) and medium (HI = 9 $\log_2$) titers of PTAs interfered with humoral immune responses to H9N2 vaccination. However, a booster dose could induce high and faster humoral immune responses even under the condition of PTAs interference.

The H9N2 IWV vaccine shows perfect efficacy in SPF chickens in laboratory, however, it often fails in the commercial broilers in the field. Several possible reasons may cause the vaccine failure. Antigenic drift is generally believed to be a major reason for the H9N2 IWV vaccine failure in the field. Mixed infection and/or concurrent infection of H9N2 AIV with other pathogens may also contribute to the vaccination failure. What's more, other factors, such as improper vaccination procedure, incorrect use of vaccine, density, condition and species of chickens, etc, may also contribute to vaccination failure. While the contribution of MDAs to the H9N2 IWV vaccine failure is paid less attention. In the present study, we explored the influence of MDAs on the efficacy of the H9N2 IWV vaccine. In order to minimize the influence of other factors on the efficacy of the H9N2 IWV vaccine, SPF chickens that received PTAs in laboratory (avoid mixed infection, concurrent infection and environmental conditions) were vaccinated with the H9N2 IWV vaccine (H514 strain) which are homologous to the virus challenge (avoid antigenic drift). Our study showed that PTAs interfered with chickens' immune response to the H9N2 IWV vaccine, which indicates that MDAs are one of the reasons for the H9N2 IWV vaccine failure in poultry. Forrest et al (Forrest et al. 2013) showed similar results that H5N2-specific MDAs reduce the generation of antibodies against H5N2 AIV. MDAs are also considered a factor for H5N1 IWV vaccine failure in broilers in Egyptian (Abdelwhab et al. 2012). The decrease in vaccine immune efficiency caused by the interference of MDAs may further harm the prevention and control of AIV in poultry (Kim et al. 2010, Maas et al. 2011). Therefore, overcoming MDAs interference is essential for preventing poultry from H9N2 AIV.

Understanding the mechanisms of MDAs interference is important for developing the next generation vaccines to overcome/bypass MDAs interference. However, few

studies have been performed on mechanisms in birds compared to in mammals. In mammals, MDAs inhibit all immune cells that react specifically to antigens and antibodies, including the germinal center B cells, plasma cells (PCs) and memory B cells (MBCs) (Bergstrom, Xu and Heyman 2017, De Vriese et al. 2010). While in avian, the mechanisms are still unclear. The present study showed that PCs were interfered by MDAs, nevertheless, a booster dose could get a high and faster humoral immune response even under the condition of MDAs interference, which indicates that the MBCs may not be interfered by MDAs in chickens. Further research needs to be done to explore the mechanisms of MDAs interpretation in avian species in the future.

Based on the present study, there are some suggestions for vaccination protocol to control H9N2 AIV in poultry. Firstly, because the HI titers are kept at a high level after booster in broiler of 14-day-old, I suggest that it is unnecessary to perform the third vaccination in broilers of 21-one-day-old according to the vaccination procedure in poultry farms mentioned above. Secondly, it is advisable to vaccinate broilers on hatchery since the second shot on their 14 days can induce a high and faster humoral immune response (maybe because of MBCs). Next, although a booster dose can induce high immune responses in SPF chickens with PTAs in laboratory, it may not so easy to get similar results in broilers in the field since several other factors may affect the vaccine efficacy mentioned above. Therefore, besides vaccination, they are also important to build a good long-term surveillance system, keep environmental hygiene, train poultry worker, etc. Finally, the conventional H9N2 IWV vaccine used in the field requires updating with new vaccines that can overcome/bypass MDAs or can be mixed with other types of vaccines in different generations.

2.4 Conclusions

Overall, our study suggests that H9N2-specific MDAs are one of the reasons for H9N2 vaccination failure in poultry. A booster dose can induce high and faster humoral immune responses even under the condition of MDAs interference. Moreover, understanding the mechanism of MDAs interference in avian species is essential to explore some new methods to tackle this problem in the future.

2.5 Methods

2.5.1 Animals and viruses

The field experiment was conducted in Biotechnology Research laboratory, Jiangsu Lihua Animal Husbandry co.LTD (JSLH), Changzhou 213168, China. Commercial chickens were hatched by the company at their premises; SPF chicken eggs used in the field experiment were purchased from Beijing Merial Vital Laboratory Animal

Technology and hatched in the laboratory of JULH. The laboratory experiment was done in SHVRI. SPF chicken eggs used in the laboratory experiment were purchased from Beijing Merial Vital Laboratory Animal Technology and hatched in the laboratory of SHVRI. All chickens were tagged and housed in high containment chicken isolators (2200mm * 860mm * 1880mm) and had full access to feed and water. The permit number was SHVRI-SZ-20200216-01.

The LPAIV H9N2 virus (A/Chicken/Shanghai/H514/2017) was used in the laboratory of SHVRI and abbreviated as H514. It was isolated and stored by the Research Team of the Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease, SHVRI. For experimental usage, the H9N2 virus was titrated in ten-day-old SPF embryonated chicken eggs (ECEs) (Beijing Merial Vital Laboratory Animal Technology Co., Ltd).

2.5.2 Vaccine and hyperimmune serum preparation

The commercial H9N2 (SS strain) water-in-oil IWV vaccine (Guangdong Wens Dahuanong Biotechnology Co., Ltd.) is generally used in the field. In laboratory experiments, H9N2 AIV (H514) ($10^{9.25}$ EID_{50}/0.1 ml) was inactivated with 1:2000 β-propiolactone (BPL) by constantly shaking for 16 h at 4℃. The residual β-propiolactone was evaporated at 37℃ for 2 h, and then 0.1 ml of the inactivated virus was inoculated to three eggs and incubated for 48 h to confirm the loss of infectivity by a Hemagglutination (HA) assay. Based on the phylogenetic analysis, these SS strain and H514 strain belong to antigenic cluster 1 and cluster 2 respectively. The inactivated H514 virus was then mixed with water-in-oil Montanide VG71($0.85g/cm^3$) adjuvant (Lone, Spackman and Kapczynski 2017) at a volume ratio of 3:7 according to the instruction. The VG71 BPL-inactivated H514) vaccine refers to the H9N2 IWV vaccine in this study.

Hyperimmune serum from commercial hens was collected in poultry farms that contain IgY against multiple antigens. The VG71 BPL-inactivated H514 vaccine (0.5 ml/time) was used to generate hyperimmune serum in SPF chickens by subcutaneously inoculating chickens three times, with a two-week interval in laboratory. This hyperimmune serum contains IgY only against H9N2 AIV.

2.5.3 Hemagglutination inhibition (HI) assay

The dynamics of antibodies were tested by the HI assay as previously described (Suarez et al. 1998). The HI titers were determined using the BPL-inactivated H514 virus. Antigen was diluted to standard 8 HA units in 50 µl. Serum samples were diluted in series of 2-fold dilutions. 0.5% chicken red blood cells (RBC) in phosphate buffer saline (PBS) were used in the HI assay.

2.5.4 Experimental design

Experiment one: The effect of MDAs on the efficacy of the commercial H9N2 IWV vaccine in poultry. Three groups of chickens were used. Each group consisted of sixteen one-day-old chickens with half commercial broilers and half SPF chickens, and one group was in one isolator. According to the company's vaccination procedure in the field, all chickens were vaccinated subcutaneously in the neck with the commercial H9N2 IWV vaccine (0.1 ml per chicken) on day one after hatching in group 1. In group 2, all chickens were immunized with 0.3 ml per chicken with the same vaccine at twenty-one-day-old. In group 3, chickens were vaccinated with PBS (0.1 ml per chicken) on day one after hatching to serve as negative control. Serum samples were collected from each chicken at 0-, 7-, 14-, 21- and 28-days post-vaccination (dpv) for the HI assay.

Experiment two: Mimicking MDAs by PTAs in SPF chickens in laboratory. Four groups of chickens were used. Hyperimmune serum with HI titers of 12 $\log_2$ against H9N2 virus was intravenously transferred into one-day-old SPF chickens (0.3 ml/chicken). Chickens that received 0.3 ml of PBS with vaccination served as positive control (group 1, n = 5). Chickens that received hyperimmune serum from SPF chickens without vaccination served as negative control (group 2, n = 5). Chickens received hyperimmune serum from commercial poultry hens in group 3 (n = 5) or SPF chickens in group 4 (n = 5). All chickens were immediately vaccinated subcutaneously in the neck with the H9N2 IWV vaccine (0.3 ml/chicken). Chickens in each group were challenged intravenously with 0.1 ml of H514 (106 EID50/0.1 ml) at twenty-eight dpv. Oropharyngeal and cloacal swabs were collected at 3- and 5-days post-challenge. Serum samples were collected from each chicken at 0-, 7-, 14-, 21-, 28-, 35- and 42- dpv for the HI assay.

Experiment three: The influence of different titers of H9N2-specific PTAs on the generation of antibodies. For this experiment, four different titers of antibodies were transferred into one-day-old SPF chickens (n = 10, 0.3 ml/chicken) and then half of these chickens from each group were vaccinated with 0.1 ml of H9N2 IWV vaccine per chicken one day later (group1: HI = 12 $\log_2$; group2: HI = 9 $\log_2$; group3: HI = 6 $\log_2$; group4: HI = 0 (PBS)). Then, all those chickens were vaccinated with 0.2 ml of the same vaccine fourteen immediately according to the vaccination procedure in the field. Serum samples were collected from each chicken at 0-, 7-, 14-, 21- and 28- dpv.

2.5.5 Virus quantification in oropharyngeal and cloacal swabs

Viral shedding was measured using EID50 as previously described (Klimov et al. 2012). Briefly, a series of 10-fold dilutions of the samples were prepared in PBS with 1 mg/ml Penicillin & 1 mg/ml Streptomycin. From each dilution 0.1 ml was

inoculated into the allantoic cavities of three ten-day-old ECEs and then the ECEs were incubated in an incubator at 37 °C for 48 h. During the incubation, the ECEs that died within 24 h post-incubation were discarded. Harvested allantoic fluid was tested for HA activity using 0.5 % chicken red blood cell (RBC) in PBS. The viral titers were calculated using the Reed & Muench method (Ramakrishnan and Muthuchelvan 2018).

2.5.6 Statistical analysis

Statistical analyses were done using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA) and SPSS 16 for Windows (SPSS Inc., Chicago, IL). The Student's t-test and Tukey test posted one-way ANOVA were used to compare the differences. $P \leqslant 0.05$ was considered to be significant.

2.6 List of abbreviations

AIV: avian influenza virus

LPAIV: low pathogenic avian influenza virus

HPAIV: high pathogenic avian influenza virus

MDAs: maternal-derived antibodies

PTAs: passively transferred antibodies

HI: hemagglutination inhibition

SPF: specific pathogen free

MBCs: memory B cells

SHVRI: Shanghai Veterinary Research Institute

JSLH: Jiangsu Lihua Animal Husbandry

H514: A/Chicken/Shanghai/H514/2017

ECEs: embryonated chicken eggs

BPL: β- propiolactone

HA: Hemagglutination

NDV: Newcastle disease virus

dpv: days post vaccination

dpc: days post challenge

PBS: phosphate buffer saline

RBC: red blood cells

BSL2: Biological Safety Level 2

2.7 Declarations

2.7.1 Ethics approval and consent to participate

All animal studies adhered to regulations of Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China, the Netherlands and the European Union, and were proved by the institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute (SHVRI). All

experiments involving H9N2 AIV were carried out in the Biological Safety Level 2 (BSL2) facility at the Animal Centre of SHVRI.

2.8 Consent for publication

Not applicable

2.9 Availability of data and materials

Data will be shared upon request by the readers.

2.10 Funding

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2.12 Competing interests

The author declares that he/she has no competing interests. Author Zejun Li was not involved in the journal's review or decisions related to this manuscript.

2.13 Authors' contributions

XP, ZL and MJ conceived of the study and participated in its design and coordination. HZ provided the field condition and working environment. XP, PD and JZ performed the field work. XP, XS, HC, DY, QT, XL participated in laboratory work. XP drafted the manuscript and QL modified it, ZL, MJ and QL directed the project. All authors have read and approved the final version of the manuscript

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3

Immunoadjuvant efficacy of CpG plasmids for H9N2 inactivated vaccine in chickens with maternal antibodies

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Abstract

Maternal-derived antibodies (MDAs) are one of reasons why vaccination with the H9N2 inactivated whole virus (IWV) vaccine failed in poultry. Unmethylated CpG motif-containing oligodeoxynucleotides (CpG ODN) shows great potential to overcome MDAs interference in mammals, but whether it has similar characteristics in poultry is still unknown. In the present study, different classes, and various copies of CpG ODN motifs were cloned into two different plasmids (pCDNA3.1 or T vector). Immunomodulatory activities and immunoadjuvant efficacy of these CpG ODN plasmids were tested *in vitro* and *in vivo* in the presence of passively transferred antibodies (PTAs) that were used to mimic MDAs. Results showed that the T vector enriched with 30 copies of CpG-A ODN and 20 copies of CpG-B ODN (T-CpG-AB) significantly up-regulated mRNA expression of chicken-interferon-α (ch-IFN-α), chicken-interferon-β (ch-IFN-β) and chicken-interleukin-12 protein 40 (ch-IL-12p40). When administered as adjuvant of the H9N2 IWV vaccine, the minimal doses of T-CpG-AB plasmid was 30 μg per one-day-old chicken, which could induce strong humoral immune responses in the presence of PTAs. Furthermore, T-CpG-AB plasmid-based vaccine triggered both strong humoral immune responses and cytokinesis expression in the presence of PTAs in chickens. Overall, our findings suggest that T-CpG-AB plasmid can be an excellent adjuvant candidate for the H9N2 IWV vaccine to overcome MDAs interference in chickens.

Keywords: Maternal-derived antibodies (MDAs), Passively transferred antibodies (PTAs), CpG containing oligodeoxynucleotides (CpG ODN), H9N2 avian influenza, Vaccine adjuvant

3.1 Introduction

H9N2 subtype avian influenza virus (AIV) is the most widespread and harmful low pathogenicity avian influenza virus (LPAIV), posing enormous damage to economies and public health. Vaccination is the main strategy for controlling H9N2 AIV in poultry, and H9N2 inactivated whole virus (IWV) vaccine is currently wildly used in the control of H9N2 AIV in poultry. However, most vaccination programs against H9N2 AIV have been proved to be ineffective in protection against infection or transmission in poultry although those vaccines have been claimed to work well in laboratory (Cui et al. 2021, Bahari et al. 2015, Gu et al. 2017, Peacock et al. 2019). Previous studies have demonstrated that maternal-derived antibodies (MDAs) interfere with the efficacy of the H9N2 IWV vaccine and are one of the reasons for H9N2 vaccination failures in poultry (Pan et al. 2022). Therefore, it is essential to develop novel adjuvants for the H9N2 IWV vaccine to overcome MDAs interference in poultry.

Unmethylated CpG motif-containing oligodeoxynucleotides (CpG ODN) has shown great potential as a vaccine adjuvant candidate to overcome MDAs interference in mammals. CpG ODN is the agonist of toll-like receptor 9 (TLR-9), which can induce an anti-viral response. In cotton rats, when CpG ODN and poly IC are used together as adjuvants of the inactivated measles vaccine, high titers of measles virus (MeV)-specific antibodies (both neutralizing and non-neutralizing antibodies) can be generated even in the presence of MeV-specific MDAs (Kim and Niewiesk 2013). However, it is unclear whether CpG ODN has similar characteristics in poultry.

CpG ODN has also been used as adjuvant in different vaccines against poultry viruses and bacteria triggering both cellular and humoral immune responses to vaccines (Gupta et al. 2014, Krieg and Davis 2001, Gupta et al. 2013). CpG ODN with inactivated Newcastle disease virus (NDV) or with H5N1 AIV inactivated vaccine by the intramuscular route, significantly increased serum IgY level, reducing virus shedding and triggering the production of high levels of cytokines such as chicken interferon-α (ch-IFN-α) and chicken interferon-γ (ch-IFN-γ) in specific pathogen-free (SPF) chickens (Wang et al. 2009, Linghua, Xingshan and Fengzhen 2007). In addition, CpG ODN as vaccine adjuvant can protect chickens against Newcastle disease (Zhang, Tian and Zhou 2007), AIV (Mallick et al. 2012) and infectious bursal disease virus (Wang et al. 2003). However, whether CpG ODN can help the H9N2 IWV vaccine overcome MDAs interference in poultry is still unknown.

The final MDAs transferred from dams have a high degree of variability in individual broilers (Gharaibeh, Mahmoud and Al-Natour 2008), which makes it difficult to study MDAs-related research. However, hyperimmune serum, which contains mostly IgY,

has isotype proportions similar to MDAs and can therefore be used to mimic the presence of MDAs in SPF chickens (Hamal et al. 2006, Faulkner et al. 2013, Pan et al. 2022). Therefore, in this study, we used passively transferred antibodies (PTAs) as a model to mimic MDAs in one-day-old SPF chickens. In addition, various classes and copies of CpG ODN were evaluated for the immunoadjuvant activity when used in combination with the H9N2 IWV vaccine in the presence of PTAs in chickens.

3.2 Maternal and methods

3.2.1 Ethics statement

All animal studies adhered to regulations of Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China, the Netherlands and the European Union, and were proved by the institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute (SHVRI). The permit number was SHVRI-SZ-20200416-02.

3.2.2 Animals and viruses

SPF eggs were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd and hatched in the laboratory of Shanghai Veterinary Research Institute (SHVRI). One-day-old SPF chickens were used in this study. All chickens were tagged and housed in high containment chicken isolator (2200mm * 860mm * 1880mm) and had full access to feed and water.

For developing the H9N2 IWV vaccine and hemagglutination inhibition (HI) assay, the LPAIV H9N2 virus (A/Chicken/Shanghai/H514/2017) was isolated and stored by the Research Team of the Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease, SHVRI. For experimental usage, the H9N2 virus was propagated in 10-day-old SPF embryonated chicken eggs (ECEs) (Beijing Merial Vital Laboratory Animal Technology Co., Ltd).

3.2.3 H9N2 IWV vaccine formation

The H9N2 virus (109.25 EID50/0.1 ml) was inactivated with 1:2000 β- propiolactone (BPL) by constantly shaking for 16 h at 4℃. The residual β-propiolactone was evaporated at 37℃ for 2 h, and then 0.1 ml of the inactivated virus was inoculated in three eggs and incubated for 48 h to confirm the loss of infectivity by a HA assay. The inactivated H514 virus was then mixed with water-in-oil Montanide VG71 (0.85 g/cm3) adjuvant (SEPPIC, France) at a volume ratio of 3:7 following manufacturer instructions.

3.2.4 Hemagglutination inhibition (HI) assay

For the HI assay, the H9N2 virus was used as a target antigen which was homologous to the vaccine strain used in this study and diluted to standard HA units (8 HA in 50 µl). Serum samples were diluted in a serial 2-fold dilutions and 0.5% chicken red blood was used in the HI assay.

3.2.5 Passively transferred antibodies (PTAs) model

The PTAs model was described previously to mimic MDAs in one-day-old SPF chickens (Pan et al. 2022). Hyperimmune sera containing H9N2-specific IgY was generated by subcutaneous injection of five-week-old SPF chicken with the H9N2 IWV vaccine (0.3 ml per chicken) three times, with a two-week interval. Sera (0.3 ml) containing H9N2-specific antibodies (HI = 12 $\log_2$) was transferred intravenously into a one-day-old SPF chicken to obtain antibody titers against H9N2 of approximately 9 $\log_2$, which was similar to the high titers of natural MDAs detected in poultry.

3.2.6 Construction of plasmids with different classes and various copies of CpG ODN motifs

We cloned 30 copies of CpG-A (ODN 2216) motifs (5'-GGGACGATCGTC-3'), 20 copies of CpG-B (ODN 2007) motifs (5'-TCGTCGTTTGTCGTTTTGTCGTT-3') or 30 copies of CpG-A ODN motifs + 20 copies of CpG-B ODN motifs with random base-pairs among each motif into pcDNA3.1 (5428 bp, a CMV-driven plasmid) (Stachowiak et al. 1997) and in T vector (2773 bp, a lac Z promoter-driven plasmid) (Beyotime, China). The repetitive CpG-A ODN motifs and CpG-B ODN motifs were designed in the laboratory, synthesized by the company (GENEWIZ, China), and cloned in pcDNA3.1 and T vector by one step cloning kit (Vazyme, China), and were called P-CpG-A, P-CpG-B, P-CpG-AB, T-CpG-A, T-CpG-B, and T-CpG-AB.

3.2.7 Immunomodulatory activities of constructed CpG-ODN plasmids *in vitro*

To examine the best plasmid backbone for CpG ODN motifs, chicken liver cell line (LMH) was seeded at a density of 1×10^6 cells/ml in a 12-well plate, cultured in DMEM (Biological Industries, BI) containing 10% FBS (Gibgo, USA), 1 mg/ml Penicillin & 1 mg/ml Streptomycin and 1 mg/ml Gentamicin. P-CpG-A, P-CpG-B, P-CpG-AB, pcDNA3.1, T-CpG-A, T-CpG-B, T-CpG-AB, or T vector (2 µg/well) were transfected using transfex reason one (Vazyme, China) at a ratio of 1 µg plasmid:2 µl reagent according to the instruction. Supernatant was refreshed after 3h and cells incubated with DMEM only were used as negative control group. Each treatment was performed in triplicate at 37°C in a 5% CO_2 incubator. The treated LMH cells were

collected after incubation for 8, 24 and 36 h, and the total mRNA was isolated using TIANamp Virus RNA kit (TIANGEN, China). Extracted mRNA was then immediately transcribed to cDNA using primer random 9 and M-MLV reverse transcriptase (Vazyme, China). Real-time PCR assay was performed using those resultant cDNA and Universal U Probe Master Mix V2 (Vazyme, China), to quantify the mRNA level of ch-IFN-α, ch-IFN-β, ch-IFN-γ and ch-IL-12p40.

3.2.8 The H9N2 IWV vaccine formulation with different CpG ODN plasmids

The H9N2 AIV ($10^{9.25}$ EID_{50}/0.1 ml) was inactivated as mentioned above. The inactivated H9N2 AIV was then mixed with water-in-oil Montanide VG71 (0.85g/cm^3) and different CpG ODN plasmids at a volume ratio of 3:6:1.

3.2.9 Adjuvant effects of different doses of T-CpG-AB plasmid in the presence of PTAs in chickens.

Using the PTAs model to mimic MDAs, the H9N2 IWV vaccine (0.1 ml/chicken) containing 0, 10, 20 or 30 μg of T-CpG-AB plasmid was subcutaneously inoculated into one-day-old SPF chickens (n = 3/group). Sera were collected every week after inoculation and stored at -20ºC until further use for the HI assay. Chickens were sacrificed 28 days after vaccination.

3.2.10 Adjuvant effects of different classes of T-CpG ODN plasmids in the presence of PTAs in chickens.

Using the PTAs model to mimic MDAs, the H9N2 IWV vaccine (0.1 ml/chicken) containing 30 μg of T-CpG-A, T-CpG-B or T-CpG-AB plasmid was subcutaneously inoculated into one-day-old chickens (n = 3/group). Chickens inoculated with only the H9N2 IWV vaccine or PBS with T-CpG-AB plasmid were used as negative control. Blood was collected every week after vaccination and used for serum collection prior to the HI assay or for real-time PCR analysis. Total mRNA from blood samples was isolated and immediately transcribed to cDNA to be the template in real-time PCR analysis.

3.2.11 Real-time PCR analysis

Real-time PCR analysis was performed to quantify the mRNA level of chicken-interferon-α (ch-IFN-α), chicken-interferon-β (ch-IFN-β), chicken-interferon-γ (ch-IFN-γ) and chicken-interleukin-12 protein 40 (ch-IL-12p40). The template is total cDNA (described above) from chicken liver cell line (LMH) samples *in vitro* or blood samples *in vivo*. Specific primers (**Table 1**) were designed on the website (https://eu.idtdna.com/site/account/login?returnurl=%2FPrimerQuest%2F). Chicken β-actin (ch-β-actin) served as a house-keeping gene. For each gene, the cycle

threshold (Ct) values of different treatments at each time point were normalized to the respective endogenous control, ch-β-actin, to get the ΔCt value. The difference in ΔCt value between stimulated and control group was calculated (ΔΔCt). Quantification of mRNA levels from each resultant cDNA was expressed as fold changes ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001):

Table 1. Primers and probes for real-time PCR

Gene	Sequence (5' → 3')
ch-IFN-α-F	CCTTCCTCCAAGACAACGATTAC
ch-IFN-α-probe	TTGTGGATGTGCAGGAACCAGGC
ch-IFN-α-R	AGTGCGAGTGATAAATGTGAGG
ch-IFN-β-F	CCTTGAGCAATGCTTCGTAAAC
ch-IFN-β-probe	CAACGCTCACCTCAGCATCAACAA
ch-IFN-β-R	GGAAGTTGTGGATGGATCTGAA
ch-IFN-γ-F	GTGAAGAAGGTGAAAGATATCATGGA
ch-IFN-γ-probe	TGGCCAAGCTCCCGATGAACGA
ch-IFN-γ-R	GCTTTGCGCTGGATTCTCA
ch-IL-12p40F	TGGGCAAATGATACGGTCAA
ch-IL-12p40probe	CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT
ch-IL-12p40R	CAGAGTAGTTCTTTGCCTCACATTTT
ch-β actin-F	TCCCTGGAGAAGAGCTATGAA
ch-β actin-probe	TGGTCAGGTCATCACCATTGGCAA
ch-β actin-R	CAGGACTCCATACCCAAGAAAG

3.2.12 Statistics analysis

Statistical analysis was performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA) and SPSS 16 for Windows (SPSS Inc., Chicago, IL). Significant differences were calculated using One-Way ANOVA followed by Tukey test. $P \leq 0.05$ was considered to be significant.

3.3 Results

3.3.1 The T-CpG-AB plasmid shows the best immunomodulatory activities *in vitro*

To determine the immunomodulatory activities of constructed CpG ODN plasmids and the optimal plasmid background for CpG ODN, two different plasmids, pcDNA3.1 and T vector, were generated containing various copies of different CpG ODN. These CpG ODN plasmids were transferred into the LMH cells at different time points. Real-time PCR results showed that all CpG ODN plasmids had immunomodulatory activities in LMH cells, resulting in up-regulation of mRNA

expression of the cytokines ch-IFN-α, ch-IFN-β, ch-IFN-γ and ch-IL-12p40 **(Fig. 1)**. Compared to the other plasmids, T-CpG-AB triggered the highest expression of ch-IFN-α at 8 and 36 h post-stimulation and of ch-IFN-β at 36 h post-stimulation **(Fig. 1A-B)**. The mRNA expression of ch-IFN-γ was rapidly up-regulated by the P-CpG-B plasmid at 8, and further increased at 24 h post-stimulation although the differences were not significant **(Fig. 1C)**. The T-CpG-AB plasmid was found to induce significantly higher expression of ch-IL-12p40 at 8 and 24 h post-stimulation **(Fig. 1D)**

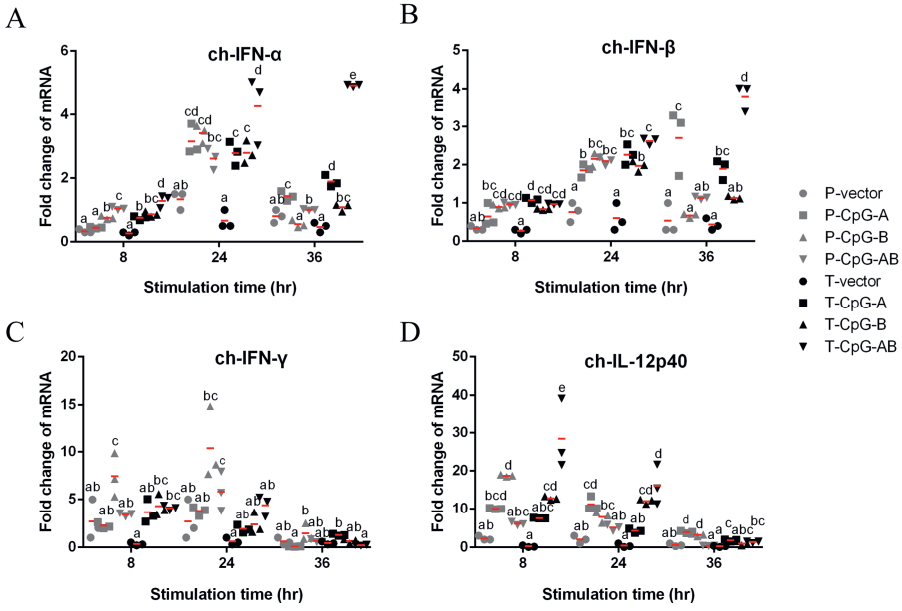

Figure 1. Immunomodulatory activities of CpG ODN plasmids on mRNA expression of cytokines *in vitro*. Different CpG ODN plasmids (2 µg) were transfected into LMH cells (1×106 cells/ml/well) and mRNA was isolated 8, 24 and 36 h post-stimulation. Non-transfected LMH cells were used as negative control. Real-time PCR was used to quantify the relative mRNA expression of (A) ch-IFN-α, (B) ch-IFN-β, (C) ch-IFN-γ and (D) ch-IFN-IL-12p40 and expressed relative to non-transfected control. Different letters denote differences among each group at the same time point. $P \leq 0.05$ was considered to be significant.

3.3.2 The minimal dose of T-CpG-AB plasmid-based adjuvant is 30 µg per chicken in the presence of PTAs

The T-CpG-AB plasmid showed the highest immunomodulatory effect *in vitro* and was therefore selected for subsequent *in vivo* experiments. To investigate whether T-CpG-AB plasmid could overcome MDAs interference when administered along with the H9N2 IWV vaccine, we used PTAs as a model to mimic MDAs in one-day-old SPF chickens. The H9N2 IWV vaccine with different doses of T-CpG-AB plasmid was inoculated subcutaneously to one-day-old chickens that also received PTAs. HI analysis showed that the H9N2 IWV vaccine when administered with 30 µg of T-CpG-AB plasmid yielded the best immune response, resulting in HI titers significantly higher than those obtained in the other groups from 21 days after vaccination onwards **(Fig. 2)**.

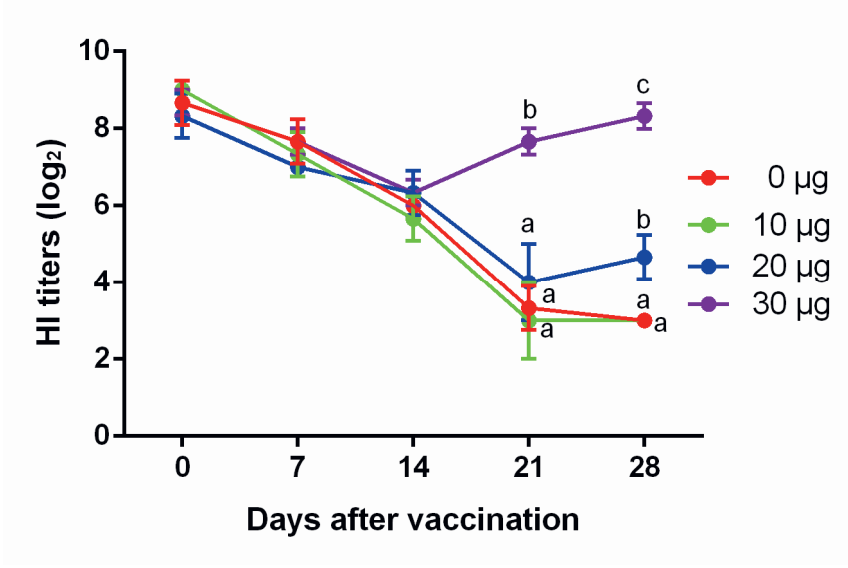

Figure 2. Administration of T-CpG-AB plasmid leads to high HI titers after vaccination with the H9N2 IWV vaccine in the presence of PTAs. One-day-old chickens (n = 3/group) with PTAs were inoculated subcutaneously with 0.1 ml of H9N2 IWV vaccines with 0, 10, 20 or 30 µg of T-CpG-AB plasmid. Different letters denote differences among each group at the same time point. $P \leq 0.05$ was considered to be significant.

3.3.3 High dose of all T-CpG ODN plasmids induces potent humoral immune responses in the presence of PTAs

The immunoadjuvant activity of the different T-CpG ODN plasmids was compared during vaccination of chickens in the presence of PTAs. One-day-old SPF chickens that received PTAs were inoculated with the H9N2 IWV vaccine and with 30 µg of the different T-CpG ODN plasmids. The HI assay was used to assess chickens' humoral immune response. The results **(Fig. 3)** showed that all T-CpG ODN plasmids, when administered with the H9N2 IWV vaccine, prevented the decline in HI titers usually observed in the presence of PTAs and, from 21 days onwards, triggered the production of antibodies leading to HI titers significantly higher than those measured in chicken that received the vaccine alone or the T-CpG-AB alone. By day 28, in all vaccinated groups receiving T-CpG ODN, HI titers reached 13 $\log_2$, a value that is considered to be protective in poultry farming.

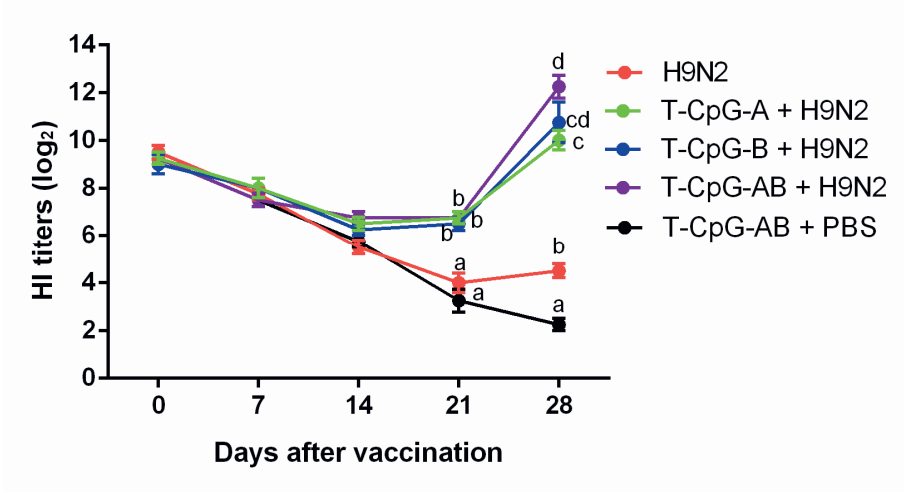

Figure 3. Administration of a single high dose of all T-CpG ODN leads to production of high HI titers after vaccination in the presence of PTAs. One-day-old chickens (n = 3/group) that received PTAs, were inoculated subcutaneously with 0.1 ml of H9N2 IWV vaccine combined with 30 µg of different classes of T-CpG ODN plasmids. Chickens inoculated with only the H9N2 IWV vaccine or PBS with T-CpG-OND plasmids were used as negative controls. Different letters denote differences among each group at the same time point.

3.3.4 The T-CpG-AB plasmid-based adjuvant triggers strong cytokine expression in the presence of PTAs

The immune response triggered by the T-CpG-ODN plasmids was evaluated also by determining mRNA expression of ch-IFN-α, ch-IFN-β, ch-IFN-γ and ch-IL-12p40 in peripheral blood leukocytes, when administered along with the vaccine in the presence of PTA. The addition of T-CpG-AB plasmid to the vaccine significantly up-regulated mRNA expression of ch-IFN-α at 21 and 28 days after vaccination **(Fig. 4A)** and ch-IFN-β at 7 days after vaccination **(Fig. 4B)**. Also, a significant up-regulation of ch-IFN-γ and ch-IL-12p40 mRNA were observed in the group receiving T-CpG-AB plasmid at 7 **(Fig. 4C)** and 14 days **(Fig. 4D)** after vaccination, respectively.

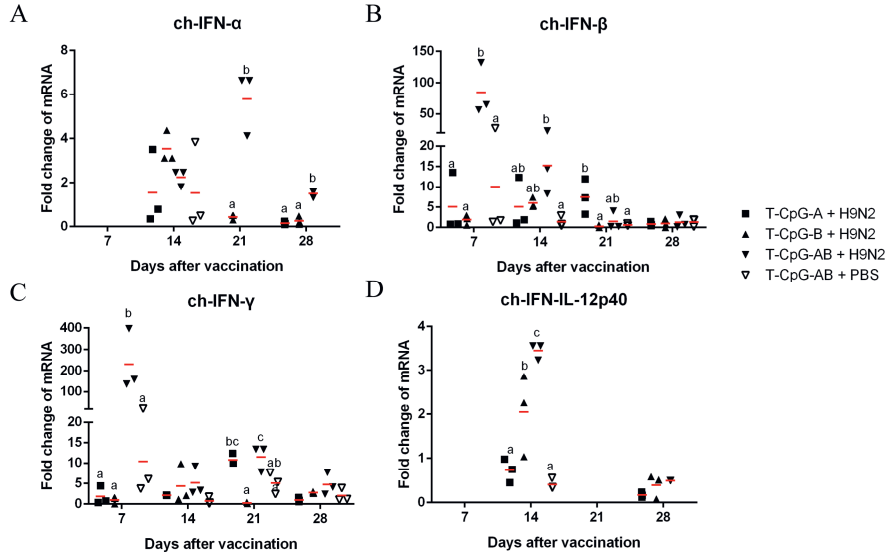

Figure 4. Immunomodulatory activities of the H9N2 IWV vaccine with different classes of T-CpG-ODN plasmids on mRNA expression of cytokines. One-day-old chickens (n = 3/group) with PTAs were inoculated subcutaneously with 0.1 ml of the H9N2 IWV vaccine with 30 μg of different T-CpG ODN plasmid. Chickens inoculated by only the H9N2 IWV vaccine or PBS with T-CpG-OND plasmids were used as negative control. The relative mRNA expression of (A) ch-IFN-α, (B)ch-IFN-β, (C) ch-IFN-γ and (D) ch-IL-12p40 were analyzed and expressed relative to the vaccine-only group. Different letters denote differences among each group at the same time point. The lack of symbols at specific time points indicates Ct values＞40, which were considered unreliable.

3.4 Discussion

MDAs are one of the reasons for the H9N2 IWV vaccine failure in poultry (Pan et al. 2022). However, few adjuvants have been reported to overcome this MDAs interference. In this study, several plasmids were generated with CpG ODN inserts. T vector containing 30 copies of CpG-A ODN motifs and 20 copies of CpG-B ODN motifs (T-CpG-AB) was found to significantly increase mRNA expression of type I ch-IFNs (ch-IFN-α and ch-IFN-β) and ch-IL-12p40 *in vitro* in chicken cell lines. To study the potential of CpG ODNs as adjuvants *in vivo*, during vaccination with the H9N2 IWV vaccine in presence of MDAs, we used PTAs as a model to mimic the presence of MDAs in SPF chickens. Under these conditions, we found that T-CpG-AB plasmid was the best adjuvant candidate, and the minimum dose was 30 μg per chicken, based on the induction of a strong humoral response and high cytokine gene expression *in vivo*. The results suggest that T-CpG-AB plasmid-based adjuvant can help the H9N2 IWV vaccine overcome MDAs interference in poultry.

H9N2 AIV is the dominant influenza strain in poultry causing huge economic losses and posing a threat to both animal and human health. The H9N2 IWV vaccine is used as a strategy to control the virus in poultry. Based on surveillance data in the field in China, broilers are generally vaccinated three times against H9N2 AIV before slaughter at approximately forty-five-day-old. However, this vaccination strategy proved not effective in controlling the virus, as it is still circulating in China. One reason for vaccination failure may be related to the high titers of H9N2-specific MDAs at the moment of vaccination. However, research focusing on improving the efficacy of vaccination in the presence of MDAs is still sparse in chickens.

CpG ODN is reported to overcome MDAs in mammals and the immunostimulatory effects of three classes (A, B and C) of synthetic CpG ODN have been well documented. CpG-A mainly acts by activating NK cells and by inducing high expression of IFN-α and IFN-β (Ballas, Rasmussen and Krieg 1996, Kadowaki, Antonenko and Liu 2001, Krug et al. 2001); CpG-B is a potent B cells activator leading to the secretion of high levels of IL-6, IL-10 and IFN-γ within a few hours after administration and leads B cells to enter G1 phase of cell cycle (Yi et al. 1996, Redford et al. 1998, Cowdery et al. 1996); CpG-C is found to activate B cells and to a lesser extend plasmacytoid dendritic cells (pDCs) (Vollmer et al. 2004, Marshall et al. 2003). In the present study, we showed that the P-CpG-B ODN plasmid (pcDNA3.1) triggered expression of ch-IFN-γ, but that T-CpG-AB plasmid triggered the highest mRNA expression of ch-IFN α, ch-IFN β and ch-IL-12p40 *in vitro*.

Although CpG ODNs show potent immunostimulatory effects and have been proven to be able to overcome MDAs interference in mammals, no study has been performed

to explore their efficacy in the presence of MDAs in chickens. The results of the present study showed that all T-CpG ODN plasmids induced high HI antibody titers when combined with the inactivated vaccine in the presence of PTAs which were used to mimic MDAs. These results, therefore, suggest that CpG ODN plasmids may overcome MDAs interference in poultry when used as adjuvant of the H9N2 IWV vaccine. In addition, CpG ODN-based adjuvant can not only enhance humoral immunity, but also significantly enhance cellular immunity by triggering strong cytokinesis expression (Hartmann, Krieg and Erinrt 1999). In the present study, the addition of T-CpG-AB plasmid during vaccination significantly increased mRNA expression of the cytokines ch-IFN-α, ch-IFN-β, ch-IFN-γ and ch-IL-12p40 in the presence of PTAs in chickens. A study in SPF chickens (Hung et al. 2011) showed similar results that a plasmid containing 64 copies of CpG ODN motifs is shown to significantly increase mRNA expression of IFN-α, IFN-γ, TLR3, TLR7 and TLR21 after vaccination with the H5N2 inactivated vaccine.

However, extensive application of CpG ODN as adjuvants for vaccines in poultry is still unfeasible. One of the hurdles is the high costs of synthesizing CpG ODN. To reduce these costs, plasmids containing multiple copies of CpG ODN motifs were created and used in the study. pcDNA3.1 and T vector plasmids were used as the backbone of CpG ODN. The results showed that CpG ODN in the T vector induced higher mRNA expression of ch-IFN-α and ch-IFN-β than in the pcDNA3.1 plasmid. This may be associated with the plasmid size. The T vector (2773 bp) is smaller than pcDNA3.1 plasmid (5428 bp), therefore, the percentage of CpG ODN part in the T vector is relatively larger. Other researchers have also successfully created T vector-based CpG ODN plasmids as adjuvant for vaccines (Fang et al. 2019, Hung et al. 2011). Other differences between these two plasmids backbone may also contribute to the different results.

The minimal dose of T-CpG-AB plasmid was also determined in the present study. We found that 30 µg of T-CpG-AB plasmid in 0.1 ml of the H9N2 IWV vaccine was the minimal dose for one-day-old chickens with high titers of PTAs (HI = 9 $\log_2$). However, another study shows that in one-day-old SPF chickens, a low dose of 2 µg per chicken of CpG-B ODN has higher immunostimulatory activities than the high dose of 20 µg per chicken when delivered intramuscularly with H9N2 inactivated virus (Singh et al. 2015). This difference may be explained by the fact that our studies are performed on chickens that received PTAs to mimic MDAs. Since MDAs interfere with immune responses, it may require more CpG ODN to get high immune responses. In addition, in our study, we used plasmids containing CpG ODN, whereas synthesized CpG ODN was used in the Singh's study.

Overall, this is the first report of an adjuvant for the H9N2 IWV vaccine to induce potent immune responses in the presence of MDAs in chickens. Various copies of CpG ODN motifs cloned into plasmids still contain immunomodulatory activities and can be used as adjuvant to overcome MDAs interference in chickens. The addition of T-CpG-AB plasmid to the vaccination is able to elicit strong humoral immune response and cytokinesis expression, even in the presence of MDAs in chickens. Generation of CpG ODN-based plasmids significantly reduces the costs compared to synthesized CpG ODN, which may make it widely applicable in poultry in the future.

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4

Efficacy of a recombinant Turkey herpesvirus (H9) vaccine in preventing transmission of homologous H9N2 virus in chickens with maternal antibodies

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Abstract

Although vaccines have been widely used for many years, H9N2 avian influenza virus (AIV) is still prevalent in China. Maternal-derived antibodies (MDAs) are purportedly a reason for H9N2 vaccination failure in poultry. The study aimed to generate a new vaccine to overcome MDAs interference to H9N2 vaccination in chickens by using mathematical models to quantify the vaccine's efficacy in stopping transmission of H9N2 AIV. Turkey herpesvirus (HVT) was used as a vaccine vector to express H9 hemagglutinin (HA) proteins. We successfully rescued and characterized the recombinant HVT expressing H9 HA proteins (rHVT-H9) in primary chicken embryonic fibroblasts (CEFs). Western blot and indirect immunofluorescence assay (IFA) showed that the rHVT-H9 consistently expressed HA proteins. In addition, the rHVT-H9 had similar growth kinetics to parent HVT. Preliminary animal experiments showed that compared to the conventional inactivated whole virus (IWV) vaccine, the rHVT-H9 stimulated robust humoral immunity in chickens with passively transferred antibodies (PTAs) that were used to mimic MDAs. Transmission experiments showed that the rHVT-H9 induced both humoral and cellular immunity in chickens with PTAs. Furthermore, the rHVT-H9 reduced the virus shedding period and decreased the reproduction ratio (R) value in chickens with PTAs after homologous challenge. However, the vaccination in this trial did not yet bring $R<1$ after vaccination. In summary, we rescued a new rHVT-H9 vaccine, which stimulated strong humoral and cellular immunity, reducing virus shedding and transmission of H9N2 AIV even in the presence of PTAs in chickens but not yet sufficiently.

Keywords: Maternal-derived antibodies (MDAs), Passively transferred antibodies (PTAs), Recombinant vaccine, Turkey herpesvirus (HVT), Immune responses, Transmission, Reproduction ratio (R)

4.1 Introduction

H9N2 avian influenza virus (AIV) is the most widespread and prevalent subtype of influenza virus, posing a serious threat to both the global poultry industry and human health. H9N2 AIV regularly spills over from birds to humans (Burke and Trock 2018, Pusch and Suarez 2018). Moreover, it donates partial or even whole sets of internal genes to other influenza viruses such as H5N1 (Guan et al. 2000), H5N6 (Shen et al. 2016, Zhang et al. 2016), H7N9 (Liu et al. 2013, Bi et al. 2015, Gao et al. 2013), H10N8 (Chen et al. 2014, Zhang et al. 2014) and H10N3 (Qi et al. 2022), that can also infect humans, posing a substantial threat to public health.

Vaccination is the main strategy for managing H9N2 AIV in poultry, however maternal-derived antibodies (MDAs) interfere with immune responses contributing to H9N2 vaccination failure. Currently, H9N2 inactivated whole virus (IWV) vaccine is predominantly used in poultry in many countries, including China (Zhang et al. 2008), Israel (Banet-Noach et al. 2007), South Korea (Lee and Song 2013), Morocco (Lau et al. 2016), Pakistan (Naeem and Siddique 2006), Egypt (Kilany et al. 2016), and Iran (Bahari et al. 2015), but the virus is still present in many locations (Liu et al. 2020). Pan et al (Pan et al. 2022) reported that one reason for H9N2 vaccination failure is the high titers of MDAs at the moment of vaccination in one-day-old chickens. Therefore, in this study, we aimed to generate a new vaccine to overcome MDAs interference in chickens.

Turkey herpesvirus (HVT) is considered a potential vector for polyvalent live vaccines in chickens to overcome MDAs interference. Compared to conventional inactivated vaccines, live vector vaccines can stimulate humoral, cellular immunity and even mucosal immunity against antigens of choice (Swayne et al. 2007, Liu et al. 2019, Suarez and Pantin-Jackwood 2017). In addition to these advantages of live vector vaccines, a unique characteristic of HVT-based vector vaccines is that they can be minimally or not at all impacted by maternal antibodies. This is probably because the cell-associated nature, the nature of the replication of the HVT vector, and the lack of expression of target antigens on the surface of infected cells or by the recombinant HVT vaccine all contribute to avoiding the MDAs interference against vector and/or target pathogens (Bublot et al. 2007, Faulkner et al. 2013, Bertran et al. 2018, Le Gros et al. 2009). Moreover, HVT-based vaccines have been shown to contain excellent safety characteristics for administration in one-day-old chickens or even for in-ovo vaccination. In addition, HVT-based vaccines are able to induce lifelong protection against Marek's disease and the target virus after a single vaccination (Sonoda et al. 2000). However, it is still unclear whether HVT, in the presence of H9N2-specific MDAs, can be used as a vector for H9 HA to induce potent immune responses to overcome MDAs interference in chickens.

The final MDAs transferred from dams have a high degree of variability in individual broilers (Gharaibeh, Mahmoud and Al-Natour 2008), which makes the study of MDAs-related vaccination inference more complex. However, a hyperimmune serum that contains mostly IgY has similar isotype proportions to MDAs and therefore can be used to mimic MDAs in specific pathogen-free (SPF) chickens (Hamal et al. 2006, Faulkner et al. 2013). Therefore, in the present study, passively transferred antibodies (PTAs) were used as a model to mimic MDAs in SPF chickens.

When assessing the efficacy of vaccines in the management of infectious diseases, especially zoonotic diseases, the ability to control the spread of viruses at the population level is more important than clinical protection and individual infection. Transmission of viruses can be assessed by the reproduction ratio (R), the average number of secondary infections caused by a single typical infected animal (Diekmann, Heesterbeek and Metz 1990). We, therefore, cloned the HA gene of H9N2 virus into the genome of HVT. The recombinant virus (rHVT-H9) was successfully rescued in primary chicken embryonic fibroblasts (CEFs) and characterized *in vitro* and *in vivo*. We used mathematical models to estimate the R value of H9N2 AIV in chickens vaccinated with the rHVT-H9 in the PTAs model. SIR transmission model is used to quantify the rHVT-H9 vaccine efficacy in reducing the transmission of H9N2 virus in the presence of PTAs in chickens.

4.2 Material and methods

4.2.1 Ethics statement

All animal studies adhered to the regulation of Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China, the Netherlands, and the European Union, and were approved by the institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute (SHVRI). All experiments involving H9N2 AIV were conducted in the Biological Safety Level 2 (BSL2) facility at the Animal Centre of SHVRI. The permit number was SHVRI-SZ-20200506-01.

4.2.2 Animals and virus

SPF eggs were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd and hatched in the laboratory of SHVRI. One-day-old SPF chickens were used in this study. All animals were tagged and housed in high containment isolator (2200mm * 860mm * 1880mm) and had unlimited access to feed and water.

The low pathogenicity avian influenza virus (LPAIV) H9N2 (A/Chicken/Shanghai/H514/2017) was used in the SHVRI laboratory, abbreviated as H514. The H514 strain is the prevalent strain in China now and was isolated and

stored by the Research Team of the Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease, SHVRI. For experimental use, the H514 was propagated in 10-day-old SPF embryonated chicken eggs (ECEs) (Beijing Merial Vital Laboratory Animal Technology Co., Ltd). The virus titers were calculated as median egg infectious doses (EID_{50}). HVT (FC126) parent strain (Okazaki, Purchase and Burmester 1970) was stored at the Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease, SHVRI.

4.2.3 The rHVT-H9 and H9N2 IWV vaccine formation

The H514 HA gene with CMV promoter was cloned into the genome of HVT. The recombinant strain (rHVT-H9) was generated by laboratory technicians using an overlapping fosmid-based system (Kim et al. 1992) in primary chicken embryonic fibroblasts (CEFs) rescued by laboratory technicians. The HVT and rHVT-H9 were both propagated separately in primary chicken embryonic fibroblasts (CEFs) and their titers were calculated as PFU in primary CEFs grown and maintained in Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 1% antibiotics and 10% fetal bovine serum (FBS), at 37 °C with 5% CO_2 atmosphere.

H9N2 (H514) ($10^{9.25}$ EID_{50}/0.1 ml) was inactivated with 1:2000 β-propiolactone (BPL) by constantly shaking for 16 h at 4°C. The residual β-propiolactone was evaporated at 37°C for 2 h, and then 0.1 ml of the inactivated virus was inoculated in three eggs and incubated for 48 h to confirm the loss of infectivity by a HA assay. The inactivated H9N2 virus was then mixed with water-in-oil Montanide VG71 (0.85 g/cm^3) adjuvant (SEPPIC, France) at a volume ratio of 3:7 following manufacturer instructions (Lone, Spackman and Kapczynski 2017).

4.2.4 Virus growth and plaque assays

The HVT and rHVT-H9 virus growth characteristics were examined by infecting CEFs *in vitro*. CEFs were seeded in 6-well plates (1 x 10^6/ml/well) and inoculated with 0.01 MOI of the HVT or rHVT-H9 per well. Cells were harvested and titrated at 24, 48, 72, 96, 120 h post-inoculation (p.i). To titrate the replication of those two viruses at different time points, CEFs were seeded in 96-well plates and inoculated with 10-fold dilutions of these viruses in three replications. On four days p.i., cytopathic effects were observed and calculated by median tissue culture infective dose ($TCID_{50}$).

4.2.5 Western blot and indirect immunofluorescence assay (IFA) analysis

We used PCR, western blot and IFA analysis to identify the expressions of HA proteins in the rHVT-H9. CEFs were seeded in 6-well plates (1 x 106/ml/well) and

inoculated with 0.01 MOI of the HVT or rHVT-H9. On four days p.i., the cells were harvested for PCR, western blot or IFA.

For PCR, total DNA was extracted from the harvested cells and used for template. Specific primers (**Table 1**) were used for PCR assay.

Table 1. Primers for PCR

Gene	Sequence (5' → 3')
H9N2-HA-F	CCTTCCTCCAAGACAACGATTAC
H9N2-HA-R	TTGTGGATGTGCAGGAACCAGGC
HVT-UL3.5-F	AGGCCGGGCGAATGGAGATGGTCGACG
HVT-UL3.5-R	GCATGACGGATCACTAACGA ATTTGCATGTACC

For western blot, the harvested cells were treated with SDS-PAGE loading buffer. H9N2 (H514) collected from embryonated chicken eggs was served as a positive control. 10 µl of samples were subjected to SDS-PAGE, and the separated proteins were electroblotted on polyvinylidene fluoride (PVDF) membranes and then blocked with 5% skimmed milk dissolved in 0.5% phosphate-buffered saline with Tween 20 (PBS-T). The membrane was probed with anti-H514 HA monoclonal antibody (2F10) cloned and conserved in the laboratory of Etiologic Ecology of Animal Influenza and Avian Emerging Viral Disease, SHVRI and then anti-mouse IgG-HRP (Sigma, USA). The HA glycoprotein bands were visualized after adding ECL detection reagents.

For IFA, the rHVT-H9 infected CEFs were washed twice with phosphate-buffered saline (PBS) on four days p.i. 4% paraformaldehyde was added to stabilize cells. The cells were permeabilized using 1% triton and blocked using 1% BSA. The cells were then incubated with 2F10 and then with fluorescence conjugated goat anti-mouse immunoglobulin G (Sigma, USA) at 37 °C while protected from light. The results were observed by inverse microscopy (magnifications 100×).

4.2.6 Preliminary animal experiments

Passively transferred antibodies (PTAs) were used as a model to mimic MDAs in SPF chickens as previously described (Pan et al. 2022). Briefly, 0.3 ml of hyperimmune serum with HI titers of 12 $\log_2$ against H9N2 were transferred intravenously into a one-day-old SPF chicken. The chicken produced antibody titers around 9 $\log_2$ similar to high titers of natural MDAs in poultry. One-day-old SPF chickens with PTAs (group 1, n = 3) or without PTAs (group 2, n = 3) were inoculated with 0.1 ml of the rHVT-H9 (5000 PFU) subcutaneously in the neck. PBS-inoculated chickens with PTAs served as negative control (group 3, n = 3). PBS-inoculated chickens without PTAs were brought in contact with vaccinated chickens without PTAs (group 4, n =

3) to discover whether the vaccine (rHVT-H9) would transmit among chickens. Sera were collected weekly and detected by HI assay.

4.2.7 Transmission animal experiments in chickens with PTAs

Three groups of chickens were used. Each group consisted of 13 one-day-old SPF chickens with PTAs applied in the same way as in the preliminary animal experiments to mimic MDAs. Chickens were subcutaneously inoculated with 0.1 ml of 5000 PFU rHVT-H9 (group 1, n = 13), H9N2 IWV vaccine (group 2, n = 13) or PBS (group 3, n = 13). Sera were collected weekly and HI titers against H514 were determined by HI assay. Three chickens in each group were sacrificed 28 days after vaccination to identify the replication of the rHVT-H9 in chickens with PTAs and to test the efficacy of cellular and mucosal immunity. Five chickens in each group were challenged with 0.1 ml of 10^6 EID_{50} of H514 virus intranasally 28 days after vaccination. The other five chickens in each group were added 24 h post-challenge (p.c). Oronasal and cloaca swabs were taken every two days until fourteen days p.c. At the end of the experiments, all animals were euthanized. The transmission animal experiments design were shown in **Table 2**.

Table 2. Experiment design to quantify the effect of the rHVT-H9 in the transmission of H9N2 AIV in chickens with PTAs

Group	Vaccination	Subgroup	Challenge/dose	Samplings/duration
		Sacrificed (n = 3)	None	Feather and spleen
1	rHVT-H9	Inoculated (n = 5)	Intranasal/H514, 10^6EID_{50}	Oronasal and cloaca swabs/ 14 days
		Contact (n = 5)	Contact	Oronasal and cloaca swabs/ 14 days
		Sacrificed (n = 3)	None	Feather and spleen
2	H9N2 IWV	Inoculated (n = 5)	Intranasal/H514, 10^6EID_{50}	Oronasal and cloaca swabs/ 14 days
		Contact (n = 5)	Contact	Oronasal and cloaca swabs/ 14 days
		Sacrificed (n = 3)	None	Feather and spleen
3	PBS	Inoculated (n = 5)	Intranasal/H514, 10^6EID_{50}	Oronasal and cloaca swabs/ 14 days
		Contact (n = 5)	Contact	Oronasal and cloaca swabs/ 14 days

4.2.8 Hemagglutination inhibition (HI) assay

The antibodies were tested by HI assay as previously described (Suarez et al. 1998). HI titers were determined using the BPL-inactivated H514 virus. Each antigen was diluted to standard 8 HA units in 50 µl. Serum samples were diluted in a series of 2-fold dilutions. 0.5% chicken red blood cells (RBC) in PBS were used in the HI assay.

4.2.9 Cellular and mucosal immunity

An ELISpot assay was performed to measure chicken-interferon-γ (ch-IFN-γ) production by splenocytes of vaccinated chickens with PTAs. Single splenocyte suspension was prepared as described above and calculated. Ch-IFN-γ ELISpot^{PLUS} kit (HRP) (MABTEGH, Sweden) was used following the manufacturer's instruction (Ariaans et al. 2009). Briefly, 96-well plates were conditioned by F12 culture medium (Gibco, USA) containing 10% FBS and splenocytes were seeded at 1×10^6 cells/ well in triplicate in F12 medium (10% FBS). The inactivated H9N2 virus (50µl/well) were used to restimulate splenocytes. Con A (10 µg/ml, Solarbio, China) stimulated splenocytes served as positive control. The plates were incubated for 48 hours in a 37℃ humidified incubator with 5% CO_2. Ch-IFN-γ was detected by incubation with biotinylated mouse-anti-Ch-IFN-γ (MT&C10-biotin) and Streptavidin-HRP (1:1000). The assay was developed by adding 100 µl of 3-amino-9-ethylcarbazole substrate buffer (Solarbio, China) in the dark until spots appeared. Spots were observed by inverse microscopy (magnifications 50x).

Lavage liquids from the nasal cavity and trachea of the three sacrificed chickens in each group were collected by repeated washing with 0.5 ml of PBS. Lavage liquids from the nasal cavity and trachea of naïve SPF chickens were used as negative control. Indirect ELISA was used to quantify H514 HA-specific ch-IgA (sIgA) as described (Chen et al. 2017). Briefly, the coating antigen was purified H514 virus (64 HA/50 µl), and the lavage liquids were used as the test sample. Goat anti-chicken IgA antibody (1:5000 in PBS, Sigma) was used as a second antibody, followed by HRP-linked rabbit anti-goat IgG (1: 10000 in PBS). The results were read at 450 nm using a microplate reader.

4.2.10 The rHVT-H9 detection and cytokine mRNA expression in splenocytes

The feathers and spleens of the three sacrificed chickens in the transmission experiments were collected. Splenocytes were prepared as previously described (Ariaans et al. 2008). Briefly, spleen tissue was squeezed through a 70 µm mesh in F12 culture medium (10% FBS) to get a single cell suspension. The rHVT-H9 DNA was extracted from chicken feathers and single splenocyte suspension and used for PCR assay to detect the rHVT-H9 strain. Specific primers **(Table 1)** were used to identify H9N2 HA and HVT.

Real-time PCR assay was used to detect cytokine mRNA expressions. Total mRNA from splenocytes was extracted using TIANamp Virus RNA kit (TIANGEN, China), and then immediately transcribed to cDNA using primer random 9 and M-MLV reverse transcriptase (Vazyme, China). Real-time RT-PCR assays were performed to quantify the mRNA level of chicken-interferon-α (ch-IFN-α), chicken-interferon-β (ch-IFN-β), chicken-interferon-γ (ch-IFN-γ), and chicken-interleukin-12 protein 40 (ch-IL-12p40) using the resultant cDNA and Universal U Probe Master Mix V2 (Vazyme, China). Chicken-β-actin (ch-β-actin) was used as a housekeeping gene. Specific primers **(Table 3)** were used to amplify ch-IFN-α, ch-IFN-β, ch-IFN-γ and ch-IL-12p40 according to the instruction. Three independent experiments were conducted at different times. For each gene, the cycle threshold (Ct) values of different treatments at each time point were normalized to the respective endogenous control, ch-β-actin, to get the ΔCt value. The differences in ΔCt value between vaccinated and control group (PBS) were calculated (ΔΔCt), Quantification of mRNA levels from each resultant cDNA was expressed as fold changes ($2^{-\Delta\Delta Ct}$) (Livak and Schmittgen 2001).

Table 3. Primes and probes for real-time PCR

Gene	Sequence (5' → 3')
ch-IFN-α-F	CCTTCCTCCAAGACAACGATTAC
ch-IFN-α-probe	TTGTGGATGTGCAGGAACCAGGC
ch-IFN-α-R	AGTGCGAGTGATAAATGTGAGG
ch-IFN-β-F	CCTTGAGCAATGCTTCGTAAAC
ch-IFN-β-probe	CAACGCTCACCTCAGCATCAACAA
ch-IFN-β-R	GGAAGTTGTGGATGGATCTGAA
ch-IFN-γ-F	GTGAAGAAGGTGAAAGATATCATGGA
ch-IFN-γ-probe	TGGCCAAGCTCCCGATGAACGA
ch-IFN-γ-R	GCTTTGCGCTGGATTCTCA
ch-IL-12p40F	TGGGCAAATGATACGGTCAA
ch-IL-12p40probe	CTGAAAAGCTATAAAGAGCCAAGCAAGACGTTCT
ch-IL-12p40R	CAGAGTAGTTCTTTGCCTCACATTTT
ch-β-actin-F	TCCCTGGAGAAGAGCTATGAA
ch-β-actin-probe	TGGTCAGGTCATCACCATTGGCAA
ch-β-actin-R	CAGGACTCCATACCCAAGAAAG

4.2.11 Detection of virus from oronasal and cloaca swabs

Oronasal and cloaca swabs were collected every two days post-challenge and preserved in 0.5 ml of PBS with 10 mg/ml of Penicillin & 10 mg/ml Streptomycin. 0.1 ml of PBS from each sample was inoculated into allantoic cavities of 10-day-old embryonated chicken eggs (ECEs). An HA assay using 0.5% chicken red blood cells (RBC) in PBS was done to identify whether these samples contained the virus.

4.2.12 Mathematical models

We used a SIR stochastic model as a model to estimate the transmission of animal experiments described above. As always in the SIR model, all contact animals were defined as “susceptible” (S). All inoculated and any contact animals infected through the course of the experiments were defined as “infectious” (I) from the first day of challenge or when found to be virus positive, until the last day a positive sample was found. After an infected animal stopped shedding the virus, it was defined as "recovered" (R) and thus immune. The total number of animals N = S + I + R. If a contact animal was infected, this was defined as a “case” (C). The reproduction ratio (R) with and without vaccination is the transmission parameter for transmission between individuals (Diekmann et al. 1990). We defined the transmission rate parameter (β) for the transmission rate, βSI/N; the recovery rate parameter (α) for the recovery rate, αI. Transmission-related data (S, I, C) were collected in the transmission experiments and used to estimate the transmission parameters. R was estimated based on the formula: R = β / α. Here, α is the inverse of the average duration of the infectious period T. Hence, R = βT. In this study, chickens in 50/50 ratio (I_0 = S_0 = 5) were used in each group to gain the highest power given the experimental size (Velthuis et al. 2007). We used this to quantify the differences between the transmission among rHVT-H9, H9N2 IWV and PBS vaccinated chickens in the presence of PTAs.

4.2.13 Estimation of transmission parameters and statistical analysis

The probability that a susceptible individual becomes infected during a time interval Δt is given (Velthuis et al. 2007) as:

$$p = 1 - e^{-\beta \frac{I \Delta t}{N}} \quad (1)$$

The β was estimated using a generalized linear model (GLM) implemented for our analysis in RStudio. In order to get a linear relationship, a complementary loglog link function (ln [-ln(1-p)]) was used together with the binomial distribution as the error term in the GLM analysis.

$$\text{cloglog}(p) = \ln[-\ln(1-p)] = \ln(\beta) + \ln\left(\frac{I \Delta t}{N}\right) \quad (2)$$

In this relationship, the dependent variable (p) is the number of cases (C) divided by the binomial total number of susceptible (S), and the offset equals ln ($\frac{I\Delta t}{N}$). Ln (β), its confidence intervals, and standard error were estimated using the GLM model. Therefore, β was calculated by exponentiation. The other parameter for calculating R is the average infectious period (T) of infected animals, which was directly calculated from the data. The R and its confidence bounds were estimated from the ln (β) and its confidence, calculated by the following equations, assuming independence of ln (β) and ln (T) (Klinkenberg et al. 2002).

$$Var\,[\ln(R)] = Var\,[\ln(\beta)] + Var\,[\ln(T)] \quad (3)$$

The 95% confidence interval will be

$$\ln(R) \pm 1.96\sqrt{Var\,[\ln(R)]} \quad (4)$$

The effect of vaccination using the different vaccines was estimated by the same GLM analysis. In this model, we defined $\beta_{rHVT\text{-}H9}$ for chickens immunized with the rHVT-H9, β_{IWV} for chickens immunized with the H9N2 IWV vaccine, and $\beta_{control}$ for chickens inoculated with PBS. The dependent variable was the number of new cases C divided by S (C/S). The dummy variables indicated either the rHVT-H9 group (value 1 or 0) or the H9N2 IWV vaccine group (value 0 or 1). As groups were homogeneous, the regression coefficient c_1 (see equation below) of the dummy variable for the rHVT-H9 vaccinated chickens gave the extra (or less) transmission in the rHVT-H9 group. This also applied to c_2, but then for the H9N2 IWV vaccine group. This shows the combined effect of susceptibility and infectivity. Therefore, the equation for the model was:

$$\text{cloglog}\,(p) = \ln(\beta) + \ln(\tfrac{I\Delta t}{N}) = c_0 + c_1\,\text{Ind}_{rHVT\text{-}H9} + c_2\,\text{Ind}_{IWV} + \ln(\tfrac{I\Delta t}{N}) \quad (5)$$

$$\text{Herein, } \ln(\beta) = c_0 + c_1\,\text{Ind}_{rHVT\text{-}H9} + c_2\,\text{Ind}_{IWV} \quad (6)$$

Three βs were obtained using the estimated regression coefficients from the GLM analysis:

$\beta_{control} = e^{c0}$

$\beta_{rHVT\text{-}H9} = e^{c0+c1}$

$\beta_{IWV} = e^{c0+c2}$

4.2.14 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA) and SPSS 16 for Windows (SPSS Inc., Chicago,

IL). Significant differences were calculated using Student's *t*-test or Tukey test, posed ANOVA. $P \leq 0.05$ was considered to be significant.

4.3 Results

4.3.1 Identification and characterization of the recombinant rHVT-H9 *in vitro*

To characterize the rescued recombinant rHVT-H9, the rHVT-H9 and parent HVT were inoculated into CEFs. DNA of the rHVT-H9 and HVT and the specific primers **(Table 1)** were used for PCR analysis. The agarose and polyacrylamide gels showed that the H9 HA gene was successfully integrated into the HVT vector **(Fig. 1A)**. The sequence analysis showed no mutation and the whole recombinant sequences were fully consistent with the expectation.

Western blot and IFA were performed to confirm the expression of HA gene in the HVT vector. CEFs were inoculated with the rHVT-H9 or HVT. Cells were harvested to examine HA expression by western blot or IFA at four days p.i. H9N2 virus collected from embryonated chicken eggs was used as a positive control. The results of western blot showed that the H9 HA proteins, approximately 70 KDa, could be detected in the rHVT-H9 infected CEFs, but not in the HVT infected CEFs. The position of the HA protein was confirmed to be correct by comparison with the positive control **(Fig. 1B)**. The results of IFA confirmed the H9 HA gene expression in the rHVT-H9 in CEFs **(Fig. 1C)**.

Growth kinetics of the rHVT-H9 and parent HVT were analyzed in CEFs. Virus was inoculated into primary CEFs with 0.01 MOI per well in 6 wells-plate, and the inoculated cells were harvested at 24, 48, 72, 96 and 120 h p.i. The harvested cells were titrated into CEFs. The results showed that the recombinant rHVT-H9 had similar growth kinetics to the parent HVT in CEFs **(Fig. 1D)**.

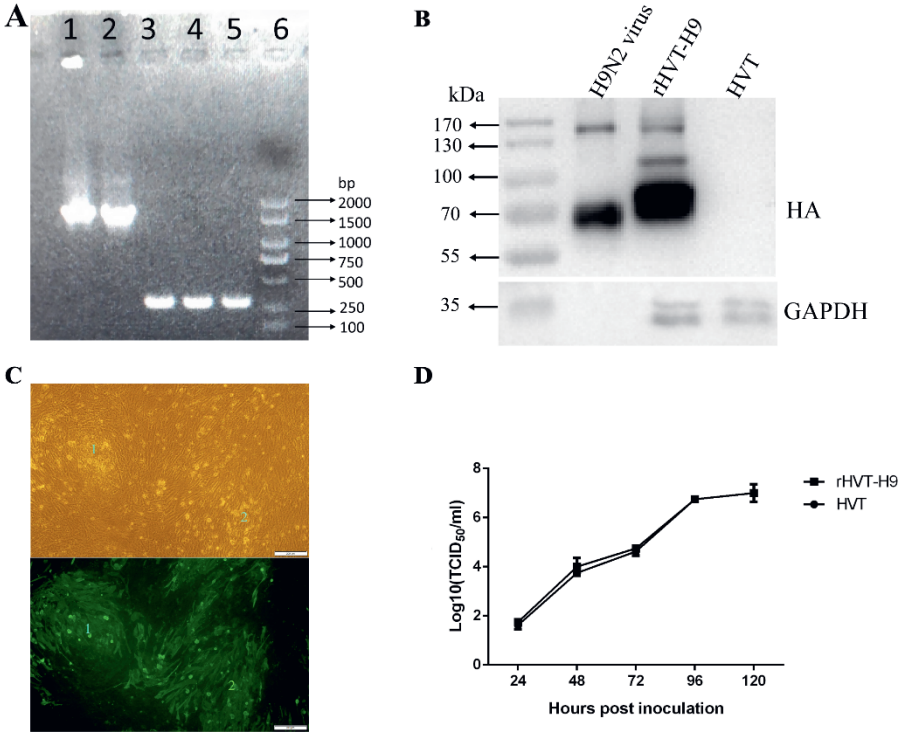

Figure.1. Detection and growth curve of the rHVT-H9 *in vitro*. (A) Gel electrophoresis of the rHVT-H9. Cytopathic CEFs were harvested to examine whether the rHVT-H9 was rescued successfully by Gel electrophoresis. Line 1 and 2 were amplified by HA-specific primers from the first and second generation of the rHVT-H9 inoculated cells respectively. Line 3 and 4 were amplified by HVT-specific primers from the first and second generation of the rHVT-H9 inoculated cells respectively. Line 5 was amplified by HVT-specific primers from the HVT inoculated cells. Line 6 was Marker 2000. (B) Western blot analysis of the rHVT-H9. CEFs were inoculated with the rHVT-H9 or HVT virus. Cells were harvested to examine HA proteins expressions by western blot at 4 days p.i. H9N2 virus collected from embryonated chicken eggs was served as positive control. (C) IFA detection of the rHVT-H9. CEFs were inoculated with the rHVT-H9. Cells were harvested to examine HA proteins expressions by IFA at 4 days p.i. The upper was observed by inverse microscopy in white light (magnifications 100×); the lower was observed by inverse microscopy in blue light (magnifications 100×). (D) Growth curve of the rHVT-H9. CEFs were inoculated with the rHVT-H9 or HVT virus with 0.01 MOI. Cells were harvested and titrated at 24, 48, 72, 96 and 120 h p.i. The error bars indicate standard deviations.

4.3.2 Humoral immune response

A preliminary animal experiment was performed to assess the safety and immunogenicity of the rHVT-H9 in chickens. The rHVT-H9 was inoculated into chickens with or without PTAs. PBS-inoculated chickens without PTA in contact with vaccinated chickens without PTA were used to explore whether the rHVT-H9 could transmit among chickens. Results showed that the vaccinated chickens with PTAs had significantly higher HI titters than PBS-inoculated chickens with PTAs, 14 days after vaccination. Vaccinated chickens with PTAs had lower HI titers than vaccinated chickens without PTAs, 21 days after vaccination. Contact SPF chickens without PTAs did not show any HI titers during the experiments **(Fig. 2A)**, which indicated that rHVT-H9 could not transmit among chickens.

To examine whether the rHVT-H9 vaccine could stop the transmission of H9N2 in the presence of MDAs in chickens, a transmission experiment was designed and performed in chickens with PTAs. Sera were collected weekly and the antibody titers against H9N2 were checked by HI assay. The results showed that the HI titers of the rHVT-H9 vaccinated chickens were significantly higher than that of the H9N2 IWV vaccine-immunized chickens with PTAs, 14 days after vaccination **(Fig. 2B)**.

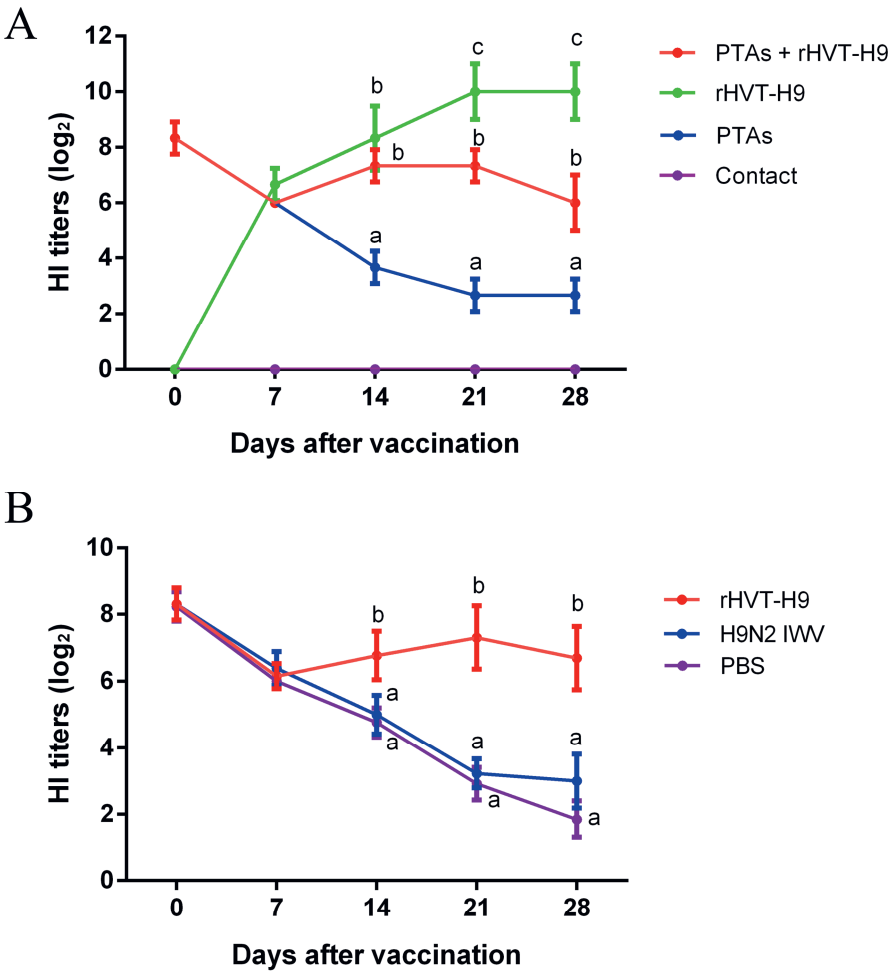

Figure.2. Humoral immune response induced in the preliminary and transmission animal experiment in chickens. (A) Sera were collected from the rHVT-H9 immunized chickens with or without PTAs (n = 3/group) weekly after vaccination and detected by HI assay. PBS-inoculated chickens with PTAs were served as negative control. Chickens without PTAs were contacted with the rHVT-H9-inoculated chickens without PTAs. (B) Sera were collected from the rHVT-H9 or H9N2 IWV vaccine-inoculated chickens with PTAs (n = 13/group) weekly after vaccination and detected by HI assay. PBS-inoculated chickens with PTAs were used as negative control. The different letters indicate significant differences between the groups at the same time point ($p < 0.05$).

4.3.3 Cellular and mucosal immune response

To examine the cellular immunity of vaccinated chickens with PTAs, a ch-IFN-γ ELISpot assay was performed following the manufacturer's instructions (Ariaans et al. 2009). The results showed that the rHVT-H9 induced high ch-IFN γ secretion which suggested potential T cell immunity **(Fig. 3B)**. This potential T cell immunity was further supported by the fact that no ch-INF-γ secretion was found in the H9N2 IWV vaccine and PBS-immunized chickens **(Fig. 3C)**.

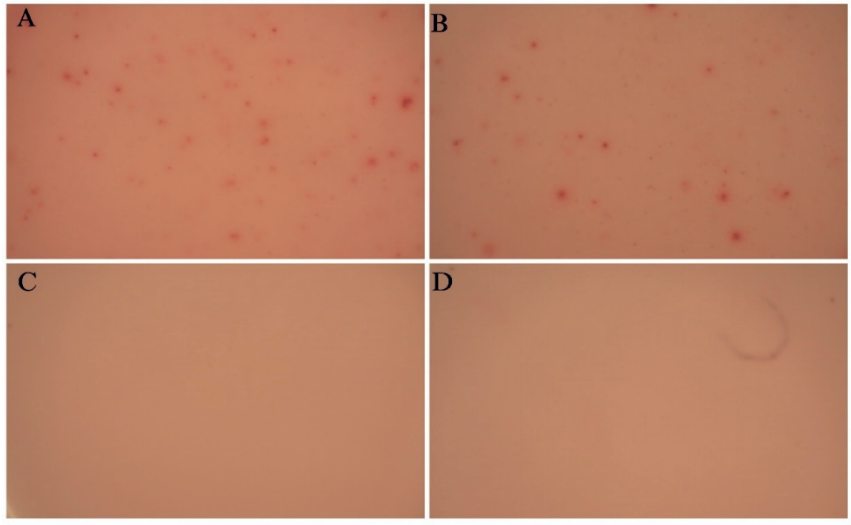

Figure.3 ELISpot assay to measure ch-IFN-γ production by splenocytes of vaccinated chickens with PTAs. Splenocytes of chickens with PTAs (n = 3/group) were harvest twenty-eight-days after vaccination. (A) Splenocytes with ConA (10 ug/ml) stimulation, (B) Splenocytes of the rHVT-H9-inoculated chickens, (C) Splenocytes of the H9N2 IWV vaccine-inoculated chickens, (D) Splenocytes of PBS-inoculated chickens. Spots were observed by inverse microscopy in white light (magnifications 50×).

To assess the efficacy of mucosal immunity, the lavage liquids from the nasal cavity and trachea of the three sacrificed chickens in each group were collected. The indirect ELISA assay showed that the ch-IgA of the rHVT-H9-immunized chickens was significantly higher than that of negative chickens. There were no significant differences among all PTAs treatment groups **(Fig. 4)**.

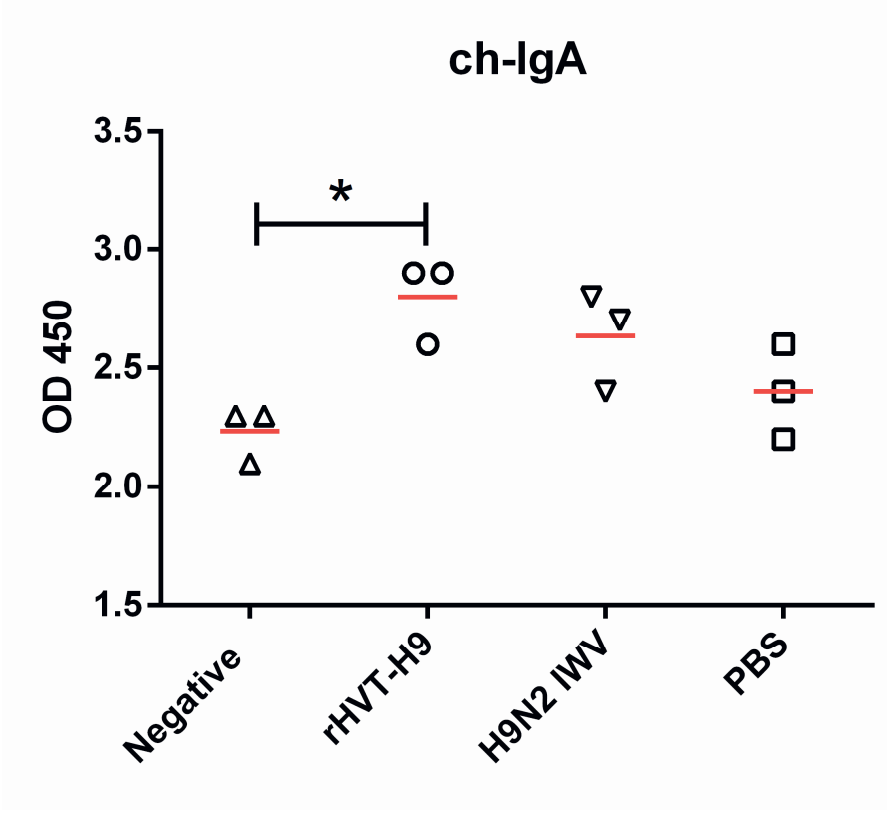

Fig.4. Mucosal immunity after vaccination in chickens with PTAs. Lavage liquids from nasal cavity and trachea of chickens with PTAs (n = 3/group) were harvest twenty-eight-day after vaccination and used to quantify H9 HA-specific ch-IgA using indirect ELISA. Symbol (*) denotes differences between two groups (* $p < 0.05$)

4.3.4 The rHVT-H9 detection and cytokines mRNA expression

To identify whether the rHVT-H9 could replicate in chickens with MDAs, three chickens from each group in the transmission experiments were sacrificed 28 days after vaccination, and their feathers and spleens were collected. Total DNA was extracted from feathers and splenocytes for H9 HA-specific PCR assay. The agarose and polyacrylamide gels results showed that all chickens vaccinated with the rHVT-H9 were positive and the positions were as expected (supplementary data). However, virus was not detected in the feathers of any group.

Cytokine mRNA expressions in splenocytes were tested by real-time PCR. All cytokines including ch-IFN-α，ch-IFN β，ch-IFN γ and ch-IL-12p40 from the rHVT-H9 vaccinated chickens were significantly higher than those from the H9N2 IWV vaccine-immunized chickens with PTAs **(Fig. 5)**.

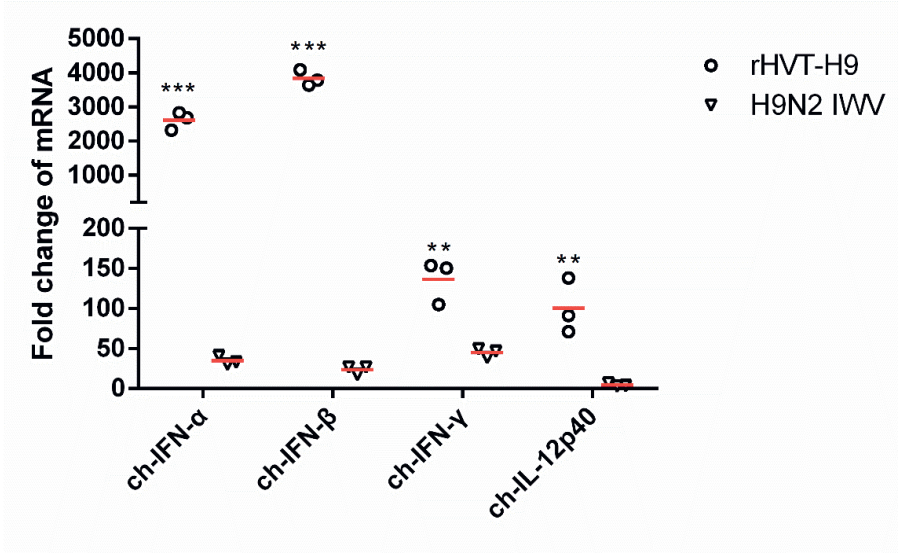

Figure.5. The mRNA expression of cytokines in splenocytes after vaccination in chickens with PTAs. Splenocytes of chickens with PTAs (n = 3/group) were collected twenty-eight-day after vaccination. Real-time PCR assay was used to detect the mRNA expressions of ch-IFN-α, ch-IFN-β, ch-IFN-γ and ch-IL-12p40. Symbol (*) denotes differences between two groups (* $p < 0.05$, ** $p < 0.01$, *** $p<0.001$).

4.3.5 Virus shedding

To identify the virus shedding in the transmission experiments, five chickens from each group (n = 10) were challenged, and another five chickens were brought in contact one day later. Oronasal and cloaca swabs were taken every two days after challenge and were detected in 10-day-old ECEs by HA assay. The results showed that the chickens immunized with the H9N2 IWV vaccine continued shedding virus until eight days p.c which was similar to PBS-inoculated chickens after challenge. However, the rHVT-H9 vaccinated chickens stopped shedding virus earlier, at four days p.c. **(Fig. 6)**.

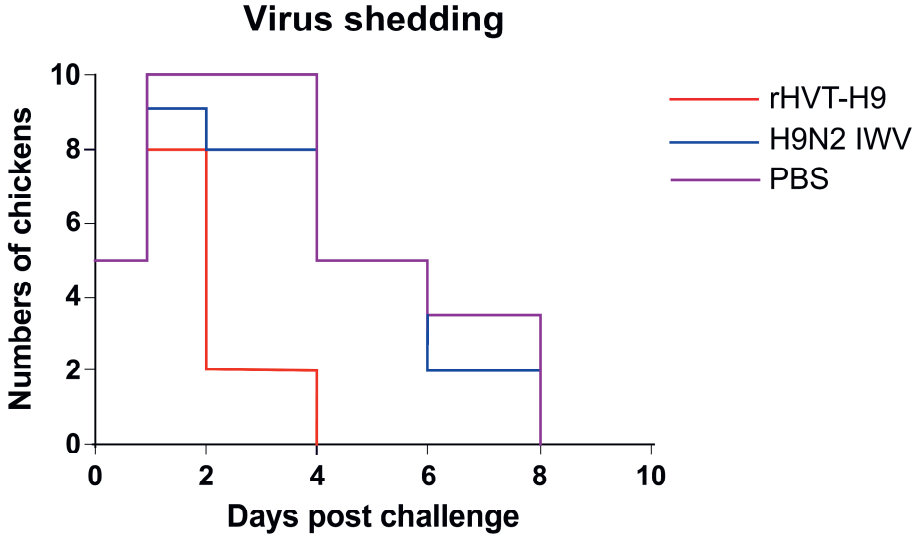

Fig.6. Numbers of virus shedding chickens after challenge. In each group (n=10), five chickens were challenged, and another five chickens contacted one day later. Oronasal and cloaca swabs were taken every two days after challenge and were detected in 10-days-old ECEs by HA assay

4.3.6 Estimation of transmission rate parameters

The transmission experiment used to quantify the transmission parameters were observed as S, I, C, and N, as described in **table 4**.

Table 4. Data abstracted from the transmission experiment for parameter estimation for the stochastic transmission model

Treatment	DS	DE	dt	N	S	I	C
rHVT-H9	1	2	1	10	5	5	4
rHVT-H9	2	4	2	10	1	8	0
rHVT-H9	4	6	2	10	1	2	0
H9N2 IWV	1	2	1	10	5	5	4
H9N2 IWV	2	4	2	10	1	9	1
PBS	1	2	1	10	5	5	5
PBS	2	4	2	10	0	10	0

DS: Day of start; DE: Day of end; dt:DE-DS; N: Total number S: Susceptible; I: Infectious; C: New cases.

GLM was used to analyze the data in R studio. The average duration of the observed shedding (T) was directly observed in these experiments (supplemental data 2). Using equation (6), the βs could be calculated. Furthermore, the corresponding R values of the different groups were estimated. The results of all the parameters were shown in **table 5**. The average infectious periods were 1.5, 4 and 5.2 in the rHVT-H9, inactivated vaccine, and control groups, respectively. The βs were 1.17 (0.47-3.44), 3.19 (1.11-9.47) and + ∞ (insufficient observations) in the rHVT-H9, inactivated vaccine, and PBS groups respectively. Therefore, R of the rHVT-H9 group was 1.75 (0.71-5.51), smaller than the R 12.76 (4.42-37.88) of the H9N2 IWV vaccine-immunized group and that of the PBS group (0.85- ∞) based on final size.

Table 5. Transmission rate parameters in different groups

Group No	Vaccine strain	Challenge strain	β (day-1)(95% CI)	infectious period (day)(95% CI)	R (95% CI)
1	rHVT-H9	H9N2 H514	1.17 (0.47-3.44)	1.5	1.75 (0.71-5.15)
2	H9N2 IWV	H9N2 H514	3.19 (1.11-9.47)	4	12.76 (4.42-37.88)
3	PBS	H9N2 H514	+ ∞	5.2	+ ∞

4.4 Discussion

MDAs are thought to be one of the reasons for the H9N2 IWV vaccine failure in poultry. To date, few vaccines have been developed to overcome the H9N2-specific MDAs interference in poultry. We successfully designed and rescued a new recombinant rHVT-H9 in CEFs. The rHVT-H9 has similar growth kinetics to parent HVT, and the expression of HA proteins were identified by PCR, western blot and IFA in vitro. Preliminary animal experiments showed that the rHVT-H9 stimulated excellent humoral immune responses compared to the H9N2 IWV vaccine. The rHVT-H9 did not transmit among chickens which indicated that it was safe as a vaccine candidate. The transmission animal experiments showed that compared to the conventional H9N2 IWV vaccine, the rHVT-H9 also induced high humoral and cellular immunity. Furthermore, the rHVT-H9-immunized group had a lower reproduction ratio (R = 1.75 (0.71,5.75)) than H9N2 IWV vaccine- and PBS-immunized group in the presence of PTAs (mimicking MDAs) in chickens. The results suggest that the rHVT-H9 can be used as a vaccine candidate to reduce transmission of H9N2 AIV in poultry.

Although MDAs provide early protection for young chickens against various diseases, they also interfere with the efficacy of vaccines. Studies in chickens show that antigen-specific MDAs interfere with the efficacy of the vaccine against H9N2 (Pan et al. 2022), H5N1(Abdelwhab et al. 2012, Maas et al. 2011), H5N2 (Forrest et al. 2013), infectious bursal disease virus (Naqi, Marquez and Sahin 1983) and Newcastle disease virus (NDV) (Van Eck, Van Wiltenburg and Jaspers 1991). As MDAs titers gradually decrease with age and interfere with vaccination efficacy, there will be a period (window) where chickens are susceptible to influenza H9N2 infections, even if a booster vaccination is given at 2-3 weeks. In the present study, we rescued a new recombinant rHVT-H9 vaccine which stimulated potential humoral and cellular immunity even in the presence of PTAs in chickens.

Live vector vaccines are good options to control pathogens as they induce not only humoral but also cellular and sometimes mucosal immunity. On the other hand, live vector vaccines are sensitive to maternal antibodies against antigens and/or vectors themselves such as Newcastle disease virus (NDV) vector vaccine (Lardinois et al. 2016, Eidson, Kleven and Villegas 1976), and fowlpox virus vector vaccine (Faulkner et al. 2013, Swayne, Beck and Kinney 2000). HVT vector vaccines against various pathogens have been previously created to tackle MDAs interference in chickens. Bublot et al (Bublot et al. 2007) showed that when using HVT as the vector for infectious bursal disease virus (IBDV), this recombinant virus protects chickens against various IBDV (mild, intermediate and hot) challenge strains despite the high titers of IBD-specific MDAs at the time of vaccination. HVT encoding the HA gene of H5N1 (rHVT-H5) offers 70% - 90% clinical protection in broilers possessing H5N1 MDAs (Rauw et al. 2012). Bertran et al (Bertran et al. 2018) also reported that

MDAs to H5N1 have minimal impact on the effectiveness of rHVT-H5. In this study, we found that when compared to the H9N2 IWV vaccine, the rHVT-H9 stimulated significantly higher antibody titers in chickens with PTAs. However, compared to SPF chickens without PTAs, chickens with PTAs had lower antibody titers after inoculation with the rHVT-H9. This indicates that the rHVT-H9 is still slightly hindered by MDAs in chickens. Studies also report that H5N1-specific MDAs interfere with the efficacy of rHVT-H5, compromising and/or delaying the generation of antibodies, although the rHVT-H5 shows protection against clinical signs and reduction of challenge virus shedding with H5N1 highly pathogenic avian influenza (HPAIVs) (Suarez and Pantin-Jackwood 2017, Kilany et al. 2015).

When assessing the efficacy of a vaccine, the best standard is not only to reduce virus shedding and provide clinical protection, but also to stop virus transmission. Although many HVT vector vaccines have been reported to overcome MDAs interference in chickens, most only focus on virus shedding and clinical protection rather than virus transmission, possibly leading to continual new infections without these being observed, also known as “silent spread”. In our study, transmission experiments were performed and analyzed using mathematical models to quantify the efficacy of the rHVT-H9 on the transmission of H9N2 AIV in chickens with PTAs. The rHVT-H9-inoculated chickens shed less virus and had a shorter shedding period compared to the H9N2 IWV vaccine- and PBS-inoculated chickens. Moreover, the R value of the rHVT-H9-inoculated groups was smaller than that of the other two groups but still >1, which indicates that the rHVT-H9 possibly alleviate but not totally stop H9N2 AIV transmission in chickens with MDAs. The uncertainty is that MDAs in the field may be more variable and lower than in our experiments. Other researchers have shown similar results that MDAs still slightly interfere with the efficacy of HVT-based vaccines (Suarez and Pantin-Jackwood 2017, Kilany et al. 2015). While a mathematical model designed by Palya et al (Palya et al. 2018) concluded that the rHVT-H5 vaccination is effective to stop HPAIV H5N8 transmission in broilers and layers, although challenging in unvaccinated layers, and vaccinated broilers and layers is not successful enough for a good comparison between groups. The different results between Palya et al ’s and our study may be because the broilers and layers used in their transmission experiments were selected without influenza-specific MDAs. In our study, chickens contained H9-specific PTAs to mimic MDAs which may interfere with immune responses. The different influenza subtypes, and in addition being highly pathogenic versus (in our case) low pathogenic may also contribute to the difference between the two studies.

T cell-mediated immunity (CMI) and local mucosal immunity are considered to be important against respiratory viruses such as influenza (Kapczynski et al. 2011, Gould et al. 2017). Liu et al (Liu et al. 2019) reported that the recombinant rHVT-H9 stimulated strong CMI and provided full protection against infection of H9N2 AIV in SPF chickens. We found strong CMI as well in the rHVT-H9-inoculated chickens

with PTAs by detection of ch-IFN-γ. However, we found that the rHVT-H9 could not provide full protection and stop transmission of H9N2 AIV in chickens with PTAs (mimicking MDAs) which were not tested in Liu's study. Insufficient information is available on mucosal immunity in chickens stimulated by HVT-based live vector vaccines. We found that there was no significant difference in mucosal immunity among the rHVT-H9, H9N2 IWV vaccine, and PBS-inoculated chickens with PTAs. The weak mucosal immunity may hinder the efficacy of the rHVT-H9 in stopping H9N2 AIV transmission in poultry.

The main strengths of the study are that 1) we are the first to assess the efficacy of the rHVT-H9 in the presence of PTAs; 2) Furthermore, we used SIR model to evaluate the ability of the rHVT-H9 in preventing transmission of H9N2 AIV instead of only infection. The results play a guiding role in clinical application in poultry, suggesting that although the rHVT-H9 is better than the conventional H9N2 IWV vaccine, it still cannot totally prevent transmission of H9N2 AIV in poultry. Therefore, we need to do further studies to improve the rHVT-H9 vaccine.

Two limitations should be noted. First, although we used PTAs to mimic MDAs in SPF chickens, it is still necessary to assess the efficacy of the rHVT-H9 in commercial chickens in poultry since the MDAs vary in individual commercial chickens. Second, we collected chickens' feathers weekly after vaccination, but didn't detect any rHVT-H9 in all feather samples. This may be because the parental strain HVT (FC126) is too mild. It is recommended to change to a more virulent strain in future studies.

In summary, we are the first to study the effect of the recombinant rHVT-H9 vaccine on reducing the transmission of H9N2 AIV in the presence of PTAs (mimicking MDAs) in chickens. The rHVT-H9 stimulated strong humoral and cellular immunity, reducing virus shedding and transmission of H9N2 AIV in the presence of PTAs in chickens but not yet sufficiently. Future rHVT-H9 studies should assess the efficacy of rHVT-H9 in commercial chickens in poultry and determine if a booster vaccination with commercial H9N2 IWV vaccine may totally prevent transmission of H9N2 AIV.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Author Contributions

XP, ZL and MJ conceived of the study and participated in its design and coordination. QL SN generated rHVT-H9. XP participated in laboratory work with the help of DH, DY, QT, XL. XP drafted the manuscript and MJ and QL modified it. ZL, MJ, NB and MF directed the project. All authors have read and approved the final version of the manuscript.

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4.5 Reference

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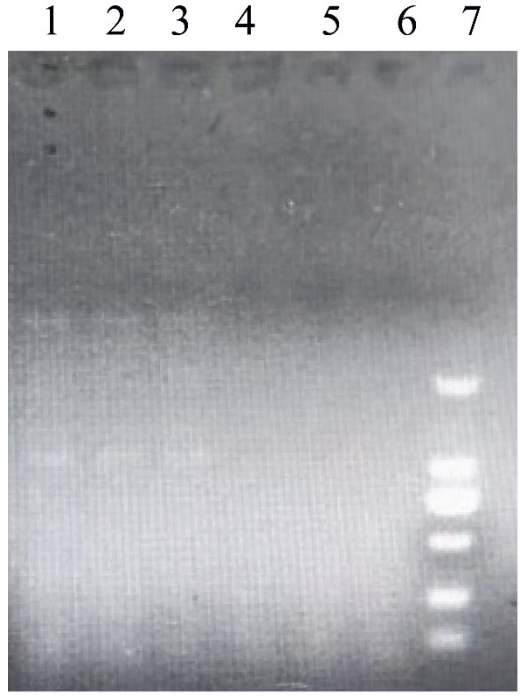
4.6 Supplementary materials



Supplementary data 1. Gel electrophoresis of the rHVT-H9 in splenocytes. Total DNA was extracted from splenocytes for H9 HA-specific PCR assay. Line 1-3 were amplified by HA-specific primers from the three rHVT-H9-immunized chickens. Line 4-6 were amplifying by HA-specific primers from the H9N2 IWV vaccine-immunized chickens. line 7 was Marker 2000.

Supplementary data 2. Overview of transmission experiment results from Day 1 p.i until Day 10 p.i.
Transmission was observed in rHVT-H9, H9N2 IWV vaccine and PBS inoculated chickens. S: Susceptible; I: Infectious; +/ or -/ when positive or negative oronasal sample; /+ or /- when positive or negative cloacal samples.

Group	Chicken	Vaccine	HI Titers (log_2)	Challenge Strain	Day 2	Day 4	Day 6	Day 8	Day 10
1	I	rHVT-H9	6	H514	+/-	-/-	-/-	-/-	-/-
1	I	rHVT-H9	6	H514	-/-	-/-	-/-	-/-	-/-
1	I	rHVT-H9	7	H514	+/+	-/-	-/-	-/-	-/-
1	I	rHVT-H9	6	H514	+/+	-/-	-/-	-/-	-/-
1	I	rHVT-H9	6	H514	+/+	-/-	-/-	-/-	-/-
1	S	rHVT-H9	6	Contact	+/-	-/+	-/-	-/-	-/-
1	S	rHVT-H9	6	Contact	+/-	-/-	-/-	-/-	-/-
1	S	rHVT-H9	6	Contact	-/-	-/-	-/-	-/-	-/-
1	S	rHVT-H9	6	Contact	+/-	-/+	-/-	-/-	-/-
1	S	rHVT-H9	6	Contact	-/+	-/-	-/-	-/-	-/-
2	I	H9N2 IWV	2	H514	+/-	+/+	-/-	-/+	-/-
2	I	H9N2 IWV	4	H514	+/-	+/-	-/-	-/-	-/-
2	I	H9N2 IWV	2	H514	+/-	+/-	-/-	-/-	-/-
2	I	H9N2 IWV	3	H514	+/-	+/-	-/-	-/-	-/-
2	I	H9N2 IWV	4	H514	+/+	-/-	-/+	-/-	-/-
2	S	H9N2 IWV	2	Contact	-/-	+/-	-/+	-/-	-/-
2	S	H9N2 IWV	4	Contact	+/-	+/-	-/-	-/+	-/-
2	S	H9N2 IWV	3	Contact	+/-	-/-	-/-	-/-	-/-
2	S	H9N2 IWV	2	Contact	+/+	+/+	-/+	-/-	-/-
2	S	H9N2 IWV	3	Contact	+/-	-/+	-/-	-/-	-/-
3	I	PBS	2	H514	+/-	+/-	-/+	-/-	-/-
3	I	PBS	1	H514	+/-	+/-	-/-	+/-	-/-
3	I	PBS	2	H514	+/+	+/+	-/-	-/-	-/-
3	I	PBS	2	H514	+/+	+/-	-/-	-/-	-/-
3	I	PBS	2	H514	+/-	+/-	-/+	-/-	-/-
3	S	PBS	2	Contact	+/+	+/-	-/+	-/+	-/-
3	S	PBS	2	Contact	+/-	+/-	+/-	-/-	-/-
3	S	PBS	3	Contact	+/+	+/+	-/-	-/-	-/-
3	S	PBS	2	Contact	+/-	+/-	-/+	+/-	-/-
3	S	PBS	2	Contact	+/-	+/-	+/-	-/+	-/-

5

Mechanisms of interference of maternal-derived antibodies with immune response to H9N2 vaccination in chickens

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Abstract

Maternal-derived antibodies (MDAs) interfere with immune responses to vaccine antigens, contributing to vaccination failure against H9N2 influenza virus in poultry. The mechanisms of MDAs interference have not been extensively investigated in chickens. This study aimed to explore the mechanisms of MDAs interference, including the identification of which portion of the MDAs interferes with immune responses to vaccination, whether chicken interferons can act as molecular adjuvants to help overcome interference, and whether T cells are affected by the presence of MDAs. To this end, we used passively transferred antibodies (PTAs) to mimic the presence of MDAs in specific pathogen-free (SPF) chickens. Homologous antibodies against H9N2 (H9N2-specific IgY), heterologous antibodies against H9N2 (H9N2-specific IgG) and homologous antibodies against Newcastle disease virus (NDV-specific IgY) were transferred intravenously into SPF chickens to mimic the complete MDAs ($F(ab)_2$ + Fc of H9N2-specific IgY), the antigen-binding portion of MDAs ($F(ab)_2$ of H9N2-specific IgG) and the Fc-binding portion of MDAs (Fc of NDV-specific). The results showed that only the complete MDAs (H9N2-specific IgY) hindered immune responses. Using the PTAs (H9N2-specific IgY) model, our data showed that intravenous injection of recombinant chicken interferons (ch-IFNs), but not intramuscular injection of plasmid-encoded ch-IFNs, elicited potent immune responses after vaccination. Flow cytometry analysis showed that the percentage of CD4+ and CD8+ T cells were not affected by PTAs (H9N2-specific IgY) during the whole experiment while. Overall, our results suggest that only the complete MDAs interfere with immune responses to vaccination, and that type I ch-IFNs can help overcome MDAs interference. In addition, MDAs do not affect T cells after vaccination.

Keywords: Maternal-derived antibodies (MDAs), Passively transferred antibodies (PTAs), chicken IFNs, H9N2 vaccination

5.1 Introduction

H9N2 subtype avian influenza virus (AIV) is the most widespread and harmful low pathogenicity avian influenza virus (LPAIV), posing enormous damage to economies and public health. Vaccination is the main control strategy against H9N2 AIV in poultry. However, although vaccines work well in laboratory, H9N2 vaccination failure happens regularly in poultry (Gu et al. 2017, Peacock et al. 2019, Bahari et al. 2015).

Maternal-derived antibodies (MDAs) are one of reasons for H9N2 vaccination failure in poultry (Pan et al. 2022). MDAs are like a double-edged sword. On the one hand, they can protect offspring from many infectious diseases at the beginning of their lives when they are vulnerable because of immature immune system (Forrest et al. 2013, Maas et al. 2011). On the other hand, MDAs may interfere with immune responses to vaccination. Although MDAs decrease with age, they still interfere with immune responses to vaccination, leading to a transient window during which animals are susceptible to infections. Therefore, vaccines need to overcome MDAs interference.

Understanding the mechanisms will contribute to overcoming the MDAs interference. However, the mechanisms of MDAs interference with immune responses to vaccination are unclear, and even in mammals, the mechanisms are still debated. Generally, five main hypotheses have been proposed: (1) antigen neutralization (Albrecht et al. 1977); (2) epitope masking (Bergstrom, Xu and Heyman 2017, Brüggemann and Rajewsky 1982, Heyman and Wigzell 1984);(3) inhibition by Fcγ receptor IIB (FcγRIIB)-mediated signaling (Kim et al. 2011, Edwards 2015); (4) clearance of MDA-coated vaccine antigens (Siegrist 2003); (5) shaping the early-life B cells repertoire (Vono et al. 2019). To date, these mechanisms of MDAs interference have been poorly investigated in avian species.

Interferons (IFNs) show great potential to overcome MDAs interference. IFNs have been shown to promote B cell survival and activation, enhancing the capacity of B cells to increase the secretion of pathogen-specific antibodies (Kiefer et al. 2012). More importantly, type I IFNs triggered by inactivated measle vaccine with Poly I:C and CpG ODN adjuvant can induce strong immune responses even in the presence of measle-specific MDAs in cotton rats (Kim and Niewiesk 2013). Nevertheless, little information is available on whether chicken interferons (ch-IFNs) can help the H9N2 inactivated whole virus (IWV) vaccine overcome MDAs interference.

MDAs interference with immune responses to vaccination has been reported in chickens for years. However, it is still unclear whether T cells are affected by MDAs. Studying MDAs-related research is difficult because the final MDAs transferred from dams have a high degree of variability in individual broilers. However, hyperimmune

serum which contains mostly IgY has similar isotype proportions to MDAs and therefore passively transferred antibodies (PTAs) can be used to mimic MDAs in specific pathogen-free (SPF) chickens (Hamal et al. 2006, Faulkner et al. 2013). Hence, in this study, we used passively transferred antibodies (PTAs) as a model to mimic the presence of MDAs in SPF chickens to explore the mechanisms of MDAs interference. Under this model, we investigated whether the antigen-binding ($F(ab)_2$), receptor-binding (Fc) or both portions of H9N2-specific MDAs interfere with immune responses to the H9N2 IWV vaccine. Furthermore, we studied whether injection of type I and type II IFNs into one-day-old chickens could stimulate potent immune responses to vaccination even in the presence PTAs (H9N2-specific IgY). Finally, we investigated whether T cells were significantly affected by the presence of PTAs (H9N2-specific IgY) during vaccination.

5.2 Material and methods

5.2.1 Ethics statement

All animal studies adhered to the regulation of Care and Use of Laboratory Animals of the Ministry of Science and Technology of the People's Republic of China, the Netherlands and the European Union, and were approved by the institutional Animal Care and Use Committee of Shanghai Veterinary Research Institute (SHVRI). The permit number was SHVRI-SZ-20200902-01

5.2.2 Animals and viruses

SPF eggs were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd and hatched in the laboratory of SHVRI. One-day-old SPF chickens were used in this study. Rabbits were purchased from Charles River, China. All animals were tagged and housed in high containment isolator (2200mm * 860mm * 1880mm) and had unlimited access to feed and water.

The low pathogenic avian influenza virus (LPAIV) H9N2, strain H514, (A/Chicken/Shanghai/H514/2017) was used as the inactivated whole virus (IWV) vaccine (as described below) and for hemagglutination inhibition (HI) assay and referred to as H9N2. This strain was isolated and stored by the research team of the Prevention of Waterfowl Viruses and Animal Influenza, Shanghai Veterinary Research Institute (SHVRI). For experimental use, the H9N2 was propagated in 10-day-old SPF embryonated chicken eggs (ECEs) (Beijing Merial Vital Laboratory Animal Technology Co., Ltd).

5.2.3 Generation of constructs, expression and quantification of recombinant chicken interferons

The gene of chicken-interferon-α (ch-IFN-α, GenBank: EU367971.1), chicken-interferon-β (ch-IFN-β, GenBank: AY974089.1) and chicken-interferon-γ (ch-IFN-γ, GenBank: AY501004.1) were retrieved from GenBank. Specific primers **(Table 1)** with homologous arms to plasmid pCAGGS and spanning the complete coding sequence (CDS) of ch-IFN-α, ch-IFN-β and ch-IFN-γ were used to amplify their transcripts by Reverse Transcriptase-PCR (TIANGEN BIORECH, China) using 1 μg total mRNA of chicken embryonic fibroblasts as a template. The full-length CDS was cloned between the *EcoRI* and *XhoI* restriction sites of pCAGGS plasmid by homologous recombination using ClonExpress Ultra One Step Cloning Kit (Vazyme, China) under the control of the chicken β-actin promoter and chimeric intron to express ch-IFNs in eukaryotic cells (Hitoshi, Ken-ichi and Jun-ichi 1991). The three plasmids were named pCAGGS-ch-IFN-α, pCAGGS-ch-IFN-β and pCAGGS-ch-IFN-γ, and were transformed in DH5α *E. coli* prior to extraction using Plasmids Maxi Kit (QIAGEN, Germany) according to the manufacturer's instruction.

Table 1. Primes spanning the complete coding sequence (CDS) of ch-IFNs with homologous arms to pCAGGS. The capital letters and lowercases indicate the homologous arms to pCAGGS or to ch-IFNs sequence, respectively.

Gene	Sequence (5' → 3')
pcaggs-ch-IFN-α F	GTCTCATCATTTTGGCAAAG atggctgtgcctgcaagcc
pcaggs-ch-IFN-α R	AGGGAAAAAGATCTGCTAGC ctaagtgcgcgtgttgcctgtga
pcaggs-ch-IFN-β F	GTCTCATCATTTTGGCAAAG atgactgcaaaccatcagtc
pcaggs-ch-IFN-β R	AGGGAAAAAGATCTGCTAGC tcactgggtgttgagacgtttgg
pcaggs-ch-IFN-γ F	GTCTCATCATTTTGGCAAAG atgacttgccagacttacaa
pcaggs-ch-IFN-γ R	AGGGAAAAAGATCTGCTAGC ttagcaattgcatctcctctgag

Human embryonic kidney cell line (HEK 293T) was seeded at a density of 1×10^7 cells/ml in T 75 flask, cultured in 15 ml serum-free opti-MEM (Biological Industries, BI) containing 1 mg/ml Penicillin & 1 mg/ml Streptomycin and 1 mg/ml Gentamicin. Each ch-IFN plasmids (10 μg) were transfected into HEK 293T cells using Transfex Reagent one (Vazyme, China) at a ratio of 1 μg plasmid:2 μl reagent according to the instruction. Supernatant was refreshed after 3h with serum-free opti-MEM and collected 48h after transfection.

The concentration of ch-IFNs in cell culture supernatants was calculated using specific chicken interferon ELISA kits (MlBio, ml-3182 for ch-IFN-α, ml-3181 for ch-IFN-β, ml-3238 for ch-IFM-γ) according to the manufacturer's instructions.

5.2.4 Relative bioactivity of recombinant ch-IFNs

The bioactivity of ch-IFNs in the cell culture supernatants of transfected HEK 293T cells was confirmed by Dual-Luciferase reporter assay system (Beyotime, China). To this end, two reporter plasmids were created. The *firefly luciferase* coding sequence (Sherf et al. 1996) was cloned in the *firefly luciferase* reporter vector pGL3c downstream of the inducible chicken Mx promoter (GenBank: EF487534.1) (called ch-IFN-αβ reporter) and of the chicken major histocompatibility complex class I (MHC I) promoter (GenBank: EU728671.1) (called ch-IFN-γ reporter) and were used to indirectly detect the presence of either ch-IFN-α and ch-IFN-β or ch-IFN-γ, respectively. Standard *Renilla luciferase* plasmid (Sherf et al. 1996), which was preserved by the research team of the Prevention of Waterfowl Viruses and Animal Influenza, SHVRI, was used to provide transfection control. Chicken embryo fibroblast cell line (DF-1) (1×10^6 /ml/well) were seeded in a 6-well plate and co-transfected using Transfex Reagent One with *Renilla luciferase* plasmids (100 ng) and ch-IFN-αβ reporter or ch-IFN- γ reporter plasmid (500 ng) at a ratio of 1 μg plasmid:2μl reagent. Supernatant was refreshed 3h after transfection. After 24h, cells were stimulated with 100 μl of HEK 293T cell culture supernatant containing ch-IFNs, and incubated for another 12h. DF-1 cells that underwent the same treatment but without incubation with ch-IFNs served as negative control. Each treatment was performed in triplicate at 37℃ in the presence of 5% CO_2.

For quantification of the relative bioactivity of each IFN, DF-1 cells were harvested and lysed by 1× Passive Lysis Buffer (PLB). Luciferase Assay Reagent II (LAR II, 100 μl) was added to 20 μl of DF-1 cell lysate into a luminometer tube and Firefly Luciferase activity was acquired. Next, 100 μl of Stop & Glo Reagent were added and Renilla Luciferase activity was acquired. The luciferase activity was measured in a turner biosystems instrument, USA. The relative bioactivity of recombinant ch-IFNs was calculated according to the following formula: (stimulated firefly luciferase/stimulated Renilla luciferase) - (control firefly luciferase/control Renilla luciferase).

5.2.5 Generation of the H9N2 IWV vaccine

H9N2 AIV (H514) ($10^{9.25}$ EID_{50}/0.1 mL) was inactivated with 1:2000 β-propiolactone (BPL) by constantly shaking for 16h at 4℃. The residual β-propiolactone was let to evaporate at 37℃ for 2 h, and then 0.1 ml of the inactivated virus was inoculated into three eggs and incubated for 48h to confirm the loss of infectivity by a hemagglutination (HA) assay. The inactivated H514 virus was then mixed with water-in-oil Montanide VG71 (0.85g/cm^3) adjuvant (SEPPIC, France) (Lone, Spackman and Kapczynski 2017) at a volume ratio of 3:7 (inactivated H514: adjuvant) according to the manufacturer's instruction.

5.2.6 Hyperimmune serum preparation and passively transferred antibodies (PTAs) model

Hyperimmune sera containing either H9N2-specific IgY or NDV-specific IgY or H9N2-specific IgG were generated by subcutaneously inoculating SPF chickens or SPF rabbits with the H9N2 IWV vaccine (0.3 ml per chicken; 0.5 ml per rabbit) or with commercial NDV inactivated vaccine (Harbin weike biotechnology, China) (0.3 ml per chicken) three times, with a two-week interval. H9N2-specific IgG was purified from the hyperimmune serum with Protein A+G Agarose (Beyotime, China).

In the present study, passively transferred antibodies (PTAs) were used as a model to mimic MDAs in SPF chickens as previously described (Pan et al. 2022). Briefly, 0.3 ml of homologous hyperimmune serum against H9N2 (H9N2-specific IgY, HI=12 $\log_2$) were transferred intravenously into one-day-old chickens, to obtain antibodies titers against H9N2 of approximately 9 $\log_2$, which is similar to the high titers of natural MDAs detected in poultry in the field.

5.2.7 Experimental design

5.2.7.1 The effect of different PTAs on the efficacy of the H9N2 IWV vaccine

One-day-old chickens (n = 4/group) received intravenously 0.3 ml of H9N2-specific IgY (group 1, HI = 12 $\log_2$), H9N2-specific IgG (group 2, HI = 12 $\log_2$), NDV-specific IgY (group 3, HI = 0), or non-specific total IgG (group 4, HI = 2 $\log_2$ which we interpreted as unspecific binding). Each of these groups mimicked either the complete MDAs ($F(ab)_2$ + Fc, group 1), or the antigen-binding site of MDAs ($F(ab)_2$, group 2), or the Fc-binding site of MDAs (Fc, group 3) or no H9N2-specific MDAs (group 4). At the same time, chickens were also vaccinated subcutaneously in the neck with 0.1 ml of the H9N2 IWV vaccine. Chickens receiving H9N2-specific IgG but not the vaccine served as negative control (group 5). Chickens in group 2 and 5 received the same dose of H9N2-specific IgG again on day 5 and 10 after vaccination. Sera were collected every week after inoculation and stored at -20ºC until further use for HI assay.

5.2.7.2 The effect of plasmid-encoded ch-IFNs on the efficacy of the H9N2 IWV vaccine in the presence of PTAs (H9N2-specific IgY)

One-day-old chickens (n = 3/group) received intravenously 0.3 ml (HI = 12 $\log_2$) of H9N2-specific IgY to mimic MDAs in SPF chickens and were intramuscularly injected in one leg with 30 μg of empty pCAGGS vector, pCAGGS-ch-IFN-α, pCAGGS-ch-IFN- β or pCAGGS-ch-IFN- γ in endotoxin-free water. Chickens were then inoculated subcutaneously in the neck with 0.1 ml of the H9N2 IWV vaccine. Ch-IFNs, in serum samples collected at the indicated time points after vaccination,

were measured by ELISA using the corresponding ch-IFNs ELISA Kit (MLBio, China) as described above. Sera were collected every week after inoculation and stored at -20ºC until further use for HI assay.

5.2.7.3 The effect of type I or type II recombinant ch-IFNs on the efficacy of the H9N2 IWV vaccine in the presence of PTAs (H9N2-specific IgY)

One-day-old chickens (n =3/group) received intravenously 0.3 ml of H9N2-specific IgY (HI=12 $\log_2$) and 0.1 ml of cell culture supernatant from cells transfected with the empty pCAGGS plasmid, or cell culture supernatant containing ch-IFN-α (1.8 ng/ml) or ch-IFN-β (1.8 ng/ml) or ch-IFN-γ (1.6 ng/ml). Chickens were also inoculated subcutaneously in the neck with 0.1 ml of the H9N2 IWV vaccine. Sera were collected every week after inoculation and stored at -20ºC until further use for HI assay.

5.2.7.4 The effect of PTAs (H9N2-specific IgY) on immune responses after vaccination with the H9N2 IWV vaccine

One-day-old chickens (n = 3/group), with or without 0.3 ml of PTAs (H9N2-specific IgY, HI=12 $\log_2$), were inoculated subcutaneously in the neck with 0.1 ml of the H9N2 IWV vaccine. Chickens with or without PTAs not inoculated with the vaccine served as the negative control. Blood was collected every week after inoculation either for peripheral blood mononuclear cells (PBMC) by flow cytometry (FCM) analysis or for collection of serum prior to HI assay.

5.2.8 Hemagglutination Inhibition (HI) Assay

The antibodies were tested by HI assay as previously described (Suarez et al. 1998). HI titers were determined using the BPL-inactivated H514 virus. Each antigen was diluted to standard 8 HA units in 50 µl. Serum samples were diluted in series of 2-fold dilutions; 0.5% chicken red blood cells (RBC) in PBS were used in HI assay.

5.2.9 Flow cytometry

A 20 µl volume of a panel of conjugated monoclonal antibody listed below, against chicken cell surface markers, was added to 100 µl of whole blood in a falcon tube and incubated in the dark for 15 min. All monoclonal antibodies were obtained from Invitrogen company, USA. Panel: anti-CD3ε-FITC (MA5-28696), anti-CD4-PE (MA5-28686) and anti-CD8-PE-Cyanine5 (MA5-28727). After incubation in the dark, 2 ml of red blood cell lysing solution (Biological Industries, China) were added to falcon tubes for 10 min at room temperature to lyse red blood cells. After washing with PBS and centrifugation for 5 min at 2000×, cells were analyzed on ACEA NovoCyte™ (BD Biosciences) flow cytometer. Raw data was performed and analyzed using Novoexpress software version 1.5.0.

5.2.10 Statistical analysis

Statistical analyses were performed using GraphPad Prism version 6.0 for Windows (GraphPad Software, San Diego, CA). Significant differences were calculated using a one-way ANOVA followed by a Tukey post-hoc test using SPSS software (Windows v16.0). $P \leq 0.05$ was considered to be significant.

5.3 Results.

5.3.1 The complete H9N2-specific IgY ($F(ab)_2$ + Fc) interferes with the efficacy of the H9N2 IWV vaccine

It has been proposed that MDAs may interfere with vaccination at various levels, either through their antigen-binding portion $F(ab)_2$, responsible for antigen neutralization, epitope masking or elimination of vaccine antigen-expressing cells, or through the Fc portion responsible for binding to inhibitory Fc-receptors on B cells. To identify which portion of MDAs interferes with the efficacy of the H9N2 IWV vaccine, different sources of antibodies, differing in their antigen specificity (H9N2 or NDV) or in their Fc portion (IgY or IgG), were passively transferred in one-day-old chickens and then vaccinated. The results showed that 21 days after vaccination, chickens that received H9N2-specific IgG and NDV-specific IgY were able to generate significantly high HI titers than chickens that received H9N2-specific IgY ($F(ab)_2$ + Fc) whose titers remained similar to those of unvaccinated chickens (**Fig.1**). Altogether these data indicated that only the antigen-binding portion, offered by H9N2-specific IgG ($F(ab)_2$), or only the Fc portion, offered by NDV-specific IgY (Fc), were not sufficient to interfere with the immune responses triggered by the H9N2 IWV vaccine. Instead, both the antigen-binding as well as Fc portion of autologous antibodies, offered by H9N2-specific IgY ($F(ab)_2$ +Fc), were required and responsible for reduced vaccine efficacy.

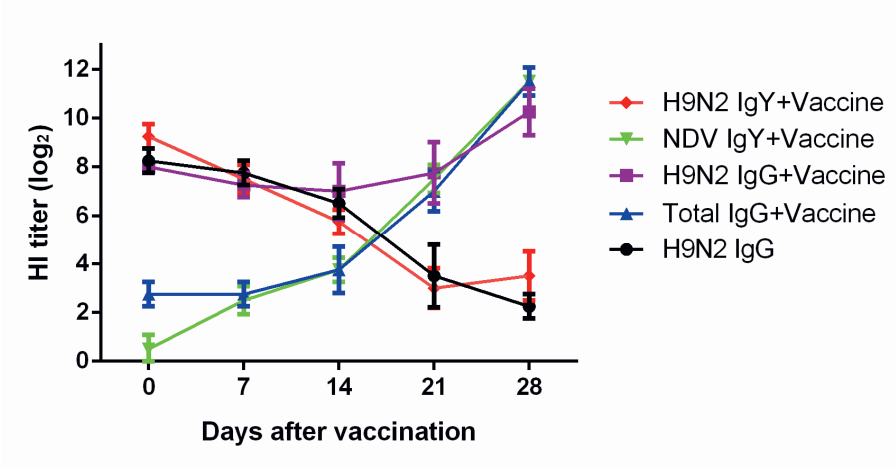

Figure 1. The HI titers of chickens received different PTAs after inoculation with the H9N2 IWV vaccine. H9N2-specific IgY, H9N2-specific IgG, NDV-specific IgY, or non-specific total IgG were injected intravenously into one-day-old chickens (n = 4/group) to mimic complete MDAs ($F(ab)_2$ + Fc), antigen-binding portion of MDAs ($F(ab)_2$), Fc portion of MDAs (Fc) and no H9N2-specific MDAs, respectively. Chickens with H9N2-specific IgG without vaccination served as negative control. Different letters denote differences ($P \leq 0.05$) among each group at the same time point.

5.3.2 Administration of plasmid-encoded ch-IFNs does not help overcome MDAs interference

Type I and type II IFNs have been shown to be positive stimulators of both B and T cell responses after vaccination as they provide co-stimulatory signals to lymphocytes that have recognized their antigen either through BCR or TCR engagement (Kim and Niewiesk 2013, Kiefer et al. 2012). We hypothesized that in the presence of MDAs, exogenous administration of type I or type II ch-IFN may help overcome MDAs interference with vaccine efficacy. To test this hypothesis, plasmids-encoded ch-IFN-α, ch-IFN-β or ch-IFN-γ (pCAGGS-ch-IFNs) were intramuscularly injected into one-day-old chickens that were vaccinated with H9N2 IWV vaccine and also received PTAs (H9N2-specific IgY). Quantification of serum levels of each of the cytokines by ELISA revealed that at 21 days after vaccination the levels of ch-IFN-α in pCAGGS-ch-IFN-α group were significantly higher than in the empty pCAGGS group **(Fig. 2A)**. Ch-IFN-β levels did not significantly change over the entire treatment period (**Fig. 2B)**. Ch-IFN-γ levels were significantly higher in pCAGGS-ch-IFN-γ group than in the empty pCAGGS group at 21 and 28 days after vaccination **(Fig. 2C)**. H9N2-specific antibody titers in pCAGGS-ch-IFN-α group were

significantly higher than in the empty pCAGGS group at 14 days after vaccination but gradually decreased owing to the natural PTAs degradation (**Fig. 2D**).

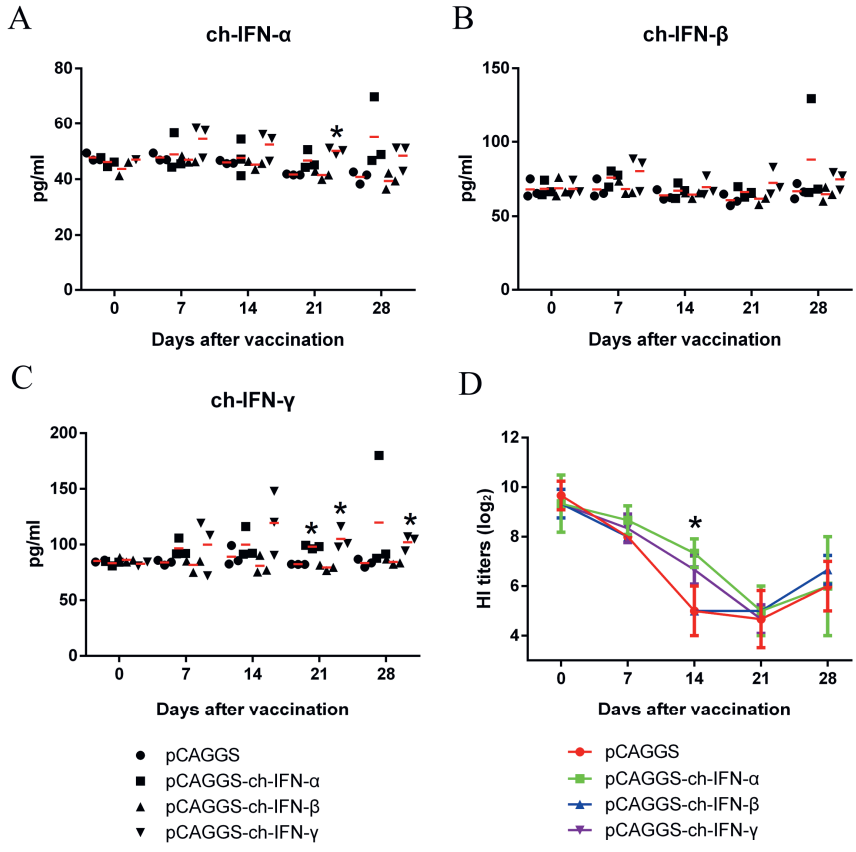

Figure 2. Administration of plasmid-encoded chicken IFNs does not improve the H9N2 IWV vaccine efficacy in the presence of PTAs (H9N2-specific IgY). pCAGGS-ch-IFN-α, pCAGGS-ch-IFN-β and pCAGGS-ch-IFN-γ were injected intramuscularly into one-day-old chickens (n = 3/group) to express ch-IFN-α (A), ch-IFN- β (B) ch-IFN- γ (C), after subcutaneous inoculation with 0.1 ml of the H9N2 IWV vaccine in the presence of PTAs (H9N2-specific IgY, HI = 12 $\log_2$). Empty pCAGGS plasmid served as negative control. (D) HI titers in each of the indicated groups. The symbol "*" indicates significant differences ($P < 0.05$) from the empty pCAGGS group at the same time point.

5.3.3 Recombinant ch-IFN-α and ch-IFN-β can overcome MDAs interference

Based on the previous data, we first wanted to exclude that the lack of effect on vaccine efficacy was due to the lack of bioactivity of the plasmid-encoded ch-IFNs. To this end, dual-Luciferase reporter assay was performed. Luciferase activity was detected in all samples which suggests that all ch-IFNs have bioactivity. The ch-IFN-α showed the highest bioactivity (**Fig. 3A**).

To determine whether recombinant ch-IFNs derived from cell culture supernatants can overcome MDAs interference to vaccination, 0.1 ml of each supernatant containing ch-IFNs were intravenously injected into one-day-old chickens that also received PTAs (H9N2-specific IgY, HI = 12 $\log_2$) and were inoculated with the H9N2 IWV vaccine. The recombinant ch-IFNs derived from cell culture supernatants were quantified by ELISA: ch-IFN-α (1.8 ng/ml), ch-IFN-β (1.8 ng/ml) and ch-IFN-γ (1.6 ng/ml). From 14 days onwards after vaccination, HI titers of chickens that received ch-IFN-α and ch-IFN-β were significantly higher than those of chickens that received ch-IFN-γ or empty pCAGGS-transfected cell supernatants (**Fig. 3B**). These data suggests that at least ch-IFN-α and ch-IFN-β may help overcome MDAs interference by boosting the immune responses to vaccination.

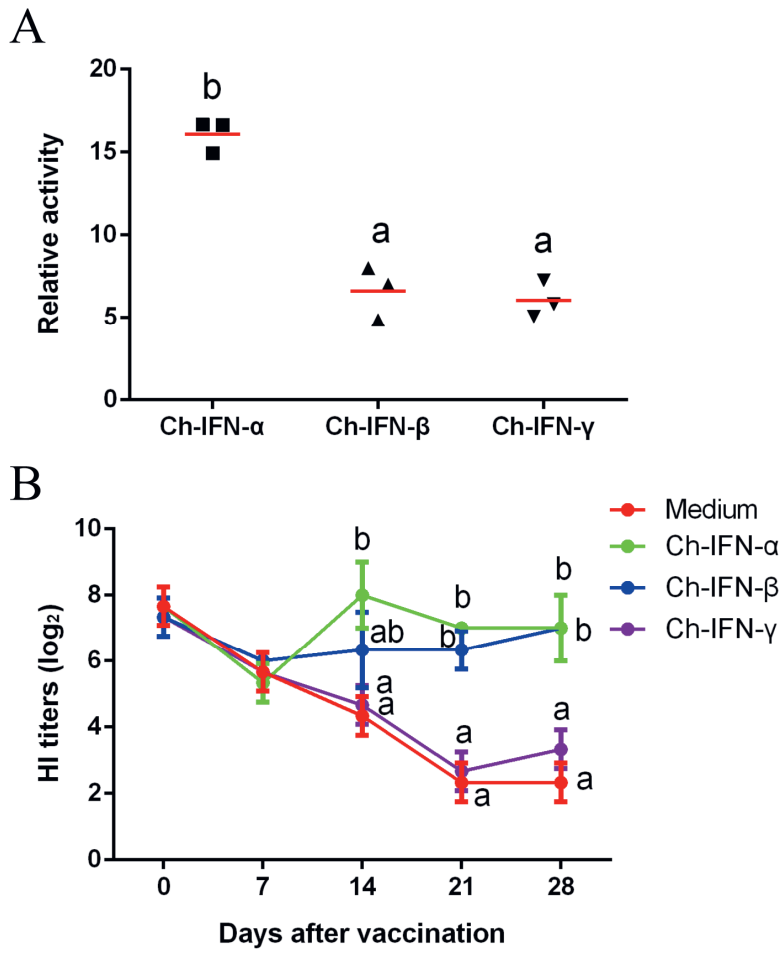

Figure 3. Recombinant ch-IFN-α and ch-IFN-β can overcome MDAs interference. (A) Relative luciferase activities: HEK 293T cells were transfected with the 10 μg of pCAGGS-ch-IFN-α, pCAGGS-ch-IFN-β and pCAGGS-ch-IFN-γ plasmid and supernatants were collected 48h later. DF-1 cells were transfected with 500 ng of ch-IFN-α, ch-IFN-β or ch-IFN- γ reporter plasmids and with 100 ng of *Renilla luciferase* reporter plasmid. Cells were stimulated or not (negative control) with 0.1 ml of the corresponding ch-IFNs-containing supernatants. (B) The HI titers of chickens (n = 3/group) that received PTAs (H9N2-specific IgY, HI = 12 $\log_2$) and ch-IFNs were tested after subcutaneous inoculation with 0.1 ml of the H9N2 IWV vaccine. Supernatant of HEK 239 T cells transfected with the empty pCAGGS was used as negative control. Different letters denote significant differences ($P < 0.05$) among each group at the same time point.

5.3.4 Flow cytometry analysis and antibody response

To identify whether T cells may be affected by the presence of MDAs, flow cytometric analysis was performed on Peripheral Blood Mononuclear Cells (PBMCs) isolated every week after vaccination. The gating strategy for lymphocytes identification was as follows **(Fig. 4)**.

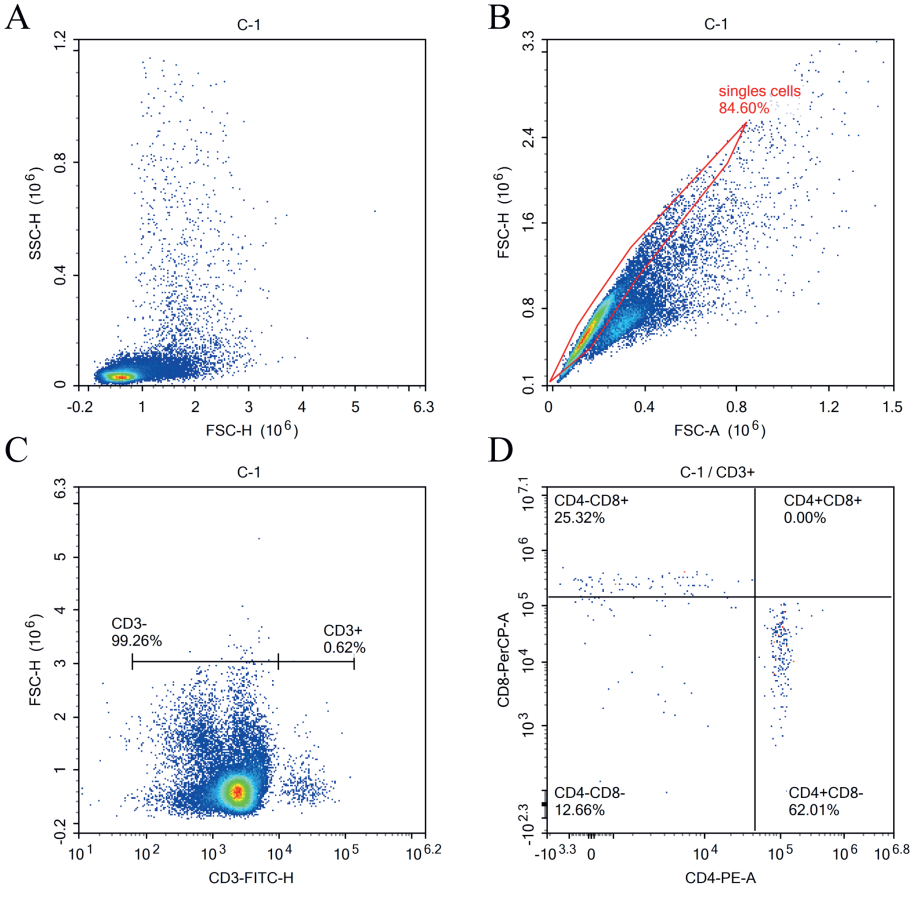

Figure 4. Gating strategy for lymphocytes identification by flow cytometry analysis. Putative lymphocytes were gated based on the light scatter properties and doublet cells were excluded based on FSC-A versus FSC-H**.** (A) T cells were identified as being $CD3^+$ and $CD4^+$ or $CD8^+$. (B) total B cells were identified as being Bu-$1a^+$ cells. The gating strategies were shown as a representative example and performed for all analyzed samples.

The percentage of $CD4^+$ and $CD8^+$ T cells in the peripheral blood was calculated based on these gating strategies using Novoexpress software version 1.5.0. software. The results showed that no significant difference in the presence of $CD4^+$ **(Fig. 5A)** and $CD8^+$ **(Fig. 5B)** T cells were found among all groups. The antibody responses were as we expected: at 21 days after vaccination vaccinated chickens that received PTAs had significantly lower HI titers than vaccinated chickens that did not receive PTAs, which were similar to PTAs group **(Fig. 5C)**.

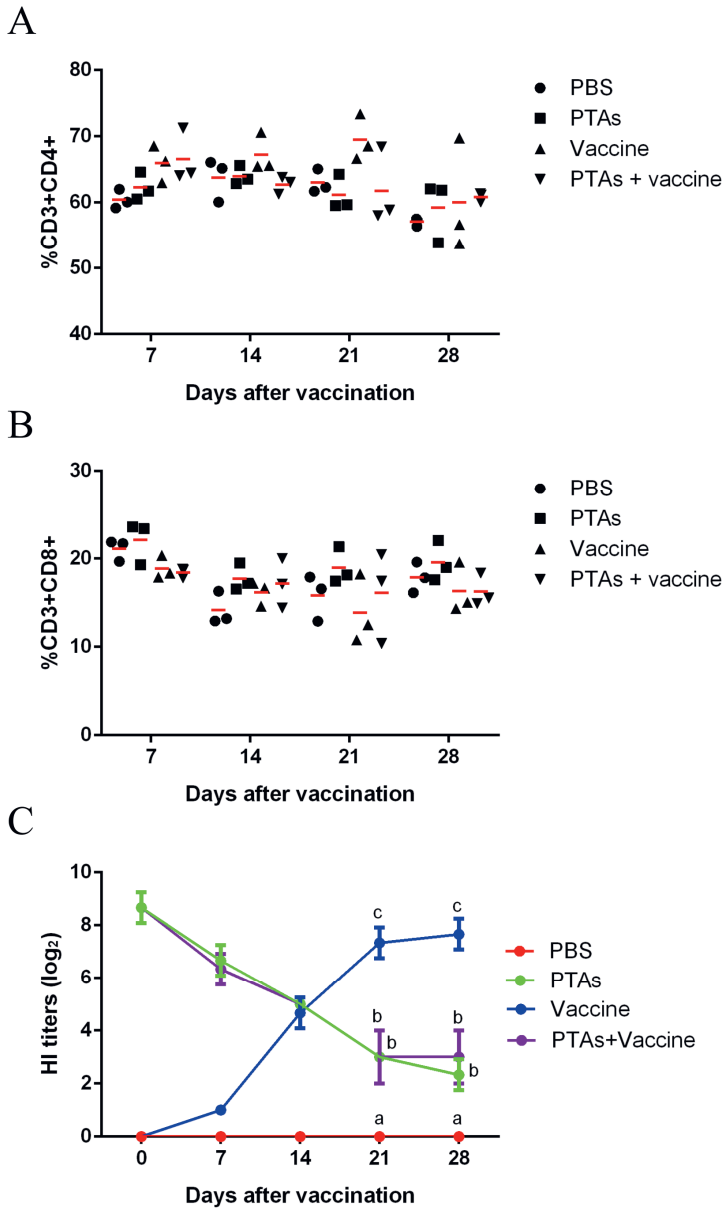

Figure 5. The presence of PTAs (H9N2-specific IgY) does not affect the relative percentage of circulating T cells after vaccination. Flow cytometry was used to quantify the relative percentage of $CD4^+$(A), $CD8^+$(B) in chicken peripheral blood (n=3/group). The HI titers were tested by HI assay (C). Different letters denote differences ($P \leq 0.05$) among each group at the same time point.

5.4 Discussion

MDAs interference with immune responses to vaccination has been well-documented in chickens. The underlining mechanisms, however, are still unclear. In the present study, through the PTAs model mimicking the presence of MDAs, four animal experiments were performed to explore the mechanisms of MDAs interference in chickens, including which portion of MDAs interfered with immune response, whether ch-IFNs could overcome MDAs interference, and whether T cells were affected by the presence of MDAs. The results showed that only complete H9N2-specific IgY ($F(ab)_2$ + Fc) interfered with immune responses instead of H9N2-specific IgG ($F(ab)_2$) or NDV-specific IgY (Fc). Intravenous injection of type I ch-IFNs into chickens, rather than administration of plasmid-encoded ch-IFNs, could overcome MDAs interference. Furthermore, the percentage of $CD4^+$ and $CD8^+$ T cells were not significantly affected by MDAs after vaccination.

It is important to understand the mechanisms of MDAs interference with immune responses. However, few studies have been performed to explore the mechanisms in avian species. The proposed mechanisms in mammals vary significantly from one study to another. In mammals, by digesting complete IgG into $F(ab)_2$ and Fc via pepsin, Kim et al (Kim et al. 2011) proved that only complete IgG interferes with immune responses. However, $F(ab)_2$ and Fc generated from digested IgG are unstable and not rigorously controlled (Niewiesk 2014). To stabilize and better control $F(ab)_2$ and Fc, we used heterologous IgG against H9N2 and homologous IgY against different antigens (H9N2 and NDV) to mimic $F(ab)_2$ and Fc respectively, and repeatedly injected heterologous IgG into chickens to avoid rapid clearance, keeping them at high levels in chicken blood. Our results are in agreement with the previous study in mammals showing that only complete H9N2-specific IgY (IgG in mammals) inhibits immune responses to vaccination.

However, some researchers doubt the conclusion that complete IgG is sufficient to mediate interference. Heyman et al (Heyman et al. 2001) found that MDAs interference occurs in both FcγRIIB-deficient and wild-type mice. Since FcγRIIB binds to the Fc portion of antibody, Heyman suggested that the Fc portion does not play a role in MDAs interference. Karlsson et al (Karlsson, Getahun and Heyman 2001) however, showed opposite results in which MDAs do not inhibit immune responses in FcγRIIB-deficient mice. In addition, some studies reported that glycosylation of the Fc is necessary for MDAs interference (Heyman, Pilström and Shulman 1988, Heyman, Nose and Weigle 1985).

In mammals, type I IFNs were shown to be crucial to overcome MDAs interference. Kim et al (Kim and Niewiesk 2013) reported that type I IFNs triggered by the

combination of Poly I:C and CpG ODN may be the key factors to overcome MDAs interference upon vaccination with MeV-inactivated vaccine. In addition, they further proved that type I IFNs can bind to complement receptor 2/CD21, leading to a strong positive signal that overcomes MDAs interference. Indeed, in mice, a high dose of IFN-α combined with the H1N1 influenza vaccine can stimulate high T cells and B cells immune responses (Proietti et al. 2002). IFN-α stimulation is shown to up-regulate B cell gene expression and to lower BCR signaling threshold (Kiefer et al. 2012), which may allow activation of the BCR signaling cascade even in the presence of MDAs. However, no study shows whether direct administration of IFNs can help overcome MDAs interference upon vaccination. This is the first study to show that intravenous injection of type I ch-IFNs, combined with the H9N2 IWV vaccine, can help overcome MDAs interference. However, intramuscular injection of plasmid-encoded ch-IFNs in the presence of PTAs cannot. This may be due to the dose (30 µg per chicken) of plasmid DNA used in the present study that may have been suboptimal. In chickens, only a high dose of DNA vaccine (10 mg per chicken), administered at least three times, confers 100% protection against infectious bursal disease in broilers having IBDV-specific maternal antibodies (Hsieh, Wu and Lin 2010).

It is still debated which lymphocyte subtype is most affected by the presence of MDAs. The current hypothesis in mammals is that MDAs interfere with B cells activation, hindering antibodies generation, and that T cells are generally not affected (Karlsson et al. 1999, Brinc et al. 2007, Getahun and Heyman 2009, Orije et al. 2020). Vono et al (Vono et al. 2019) however, reported that MDAs do not hinder B cell activation but interfere with B cell differentiation into plasma cells, which suggests that the total B cells may be not affected by MDAs. Furthermore, they found that follicular helper T (Tfh) cells prematurely decline in the presence of MDAs. Koch (Koch et al. 2016) showed that MDAs together with IgA hinder mucosal T helper cell responses in early life in mice. In this study, we found that after vaccination, the percentage of $CD4^+$ and $CD8^+$ T cells were not significantly affected. Unfortunately, we failed to calculate the percentage of total B cells in the present study because of the lack of effective B cell-specific monoclonal antibodies.

In the future, several strategies can be adopted to overcome MDAs interference in chickens. Firstly, it may help to identify new adjuvants that can stimulate high type I ch-IFNs in chickens, such as CpG ODN adjuvant as those described in Chapter 2 of this thesis. This approach has been shown to be successful in cotton rats (Kim and Niewiesk 2013) and pigs (Polewicz et al. 2013). Secondly, HVT live vector vaccine may overcome MDAs interference since the cell-associated nature, the nature of the replication of the HVT vector and the lack of expression of target antigens on the surface of infected cells or by the recombinant HVT vaccine itself, likely all contribute to circumvent MDAs interference against vector and/or target antigens (Bublot et al.

2007, Faulkner et al. 2013, Bertran et al. 2018, Le Gros et al. 2009). Next, reducing the vaccine antigen into small units (with adjuvant) is a possible approach to overcome MDAs interference, because a small antigen (30 nm) is able to trigger high immune responses in the presence of MDAs in mice (Enriquez-Rincon and Klaus 1984). This may be because small antigens cannot cross-link MDAs, BCR, and FcγRIIB, thereby blocking B cell activation. Finally, a high dose of vaccine antigen may also help overcome MDAs interference. Examples of this approach were performed in infants in the presence of MDAs (Dagan et al. 1998, Cutts et al. 1994). However, this approach should be considered carefully since a high dose of vaccine may result in unspecific mortality (Knudsen et al. 1996).

In summary, we found that only complete MDAs (H9N2-specific IgY) interfered with immune responses to vaccination. Type I ch-IFNs helped overcome MDAs interference upon vaccination with the H9N2 IWV vaccine. Our studies further demonstrated that MDAs did not affect $CD4^+$ and $CD8^+$ T cells after vaccination. Altogether, these findings may contribute to designing novel vaccination strategies aimed to overcome MDAs interference in the field, with the ultimate goal to prevent H9N2 vaccination failure in poultry.

5.5 Reference

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6

General discussion

6.1 Introduction

H9N2 avian influenza virus (AIV) is the most harmful and widespread low pathogenicity avian influenza virus (LPAIV) in the world. Vaccines have been widely used to control H9N2 AIV in poultry for many years in some countries. However, the virus is still circulating and has become endemic in several regions in spite of regular H9N2 vaccination in poultry. Thus, we may conclude that the vaccination against H9N2 has failed. Therefore, the aim of this project was 1) to figure out what factors may interfere with the H9N2 vaccine efficacy to sufficiently reduce transmission in poultry, 2) to come up with some ideas to improve the vaccine efficacy, and 3) to explore the mechanisms that influence the vaccine efficacy in poultry.

In the previous chapters of the thesis, we hypothesized that high titers of maternal-derived antibodies (MDAs) interfered with the immune responses after vaccination in poultry, and we supported this hypothesis through the use of a passively transferred antibodies (PTAs) model to mimic MDAs in specific pathogen-free (SPF) chickens. Using this model, we tested two new vaccines, that we made for this purpose: H9N2 inactivated whole virus (IWV) vaccine with CpG plasmid-based adjuvant and recombinant turkey herpesvirus influenza (H9) vaccine to overcome MDAs interference. Finally, we explored some mechanisms of MDAs interference with immune responses to vaccination with the H9N2 IWV vaccine in chickens. Based upon these findings in these papers, my discussion in this chapter will focus on 1) avian immune response after vaccination, 2) the mechanisms of MDAs interference, 3) new measurements of vaccine efficacy against transmission of H9N2 AIV, 4) strategies to improve vaccine efficacy based on new measurements.

6.2 Avian immune response after vaccination

The immune system includes innate immunity and adaptive immunity. The two parts are highly integrated, and both are important to eliminate invasive pathogens. In terms of immune responses, an ideal vaccine should have the capacity to trigger both the innate and the adaptive immune responses at the same time (Netea et al. 2019).

6.2.1 Avian innate immune response

The innate immune response provides the first line of protection against pathogens. After the invasion of a pathogen, the host pathogen recognition receptors (PRRs) will recognize and combine with pathogens' conserved components called pathogen-associated molecular patterns (PAMPs) that are shared by several pathogens, leading to the production of pro-inflammatory cytokines and antiviral molecules, helping to initiate the recruitment and the activation of inflammatory cells as well as the release of noncellular effector molecules such as complement or lysozyme (Cao 2016, Chen et al. 2018). In addition, those cytokines promote the expression of major histocompability complex (MHC) and co-stimulatory molecules in antigen-presenting cells (APCs) such as dendritic cells (DCS), regulating T helper (Th) cell differentiation and facilitating the immune responses to pathogens. The invader (pathogens) will be eliminated by phagocytosis and subsequent destruction within intracellular vesicles containing oxygen radicals and digestive enzymes. Pathogens can also be destroyed by soluble chemical factors secreted by innate immune cells.

Typically, inactivated pathogens as vaccines cannot induce innate immune responses, however, a good adjuvant for vaccines is able to stimulate strong innate immune responses triggering the secretion of complement components, chemokines and pro-inflammatory cytokines as a natural support for adaptive immunization. Toll-like receptors (TLRs) agonist-based adjuvants hold great promise as vaccine adjuvant candidates in chickens. Many TLRs agonists such as Pam3CSK4 (TLR 2) (St. Paul et al. 2014), polyinosinic-polycytidylic acid (TLR 3) (Ichinohe et al. 2005, Liang et al. 2013, Paul et al. 2012), lipopolysaccharide (TLR 4) (Paul et al. 2012, Tseng et al. 2009), bacterial flagellin (TLR 5) (Chaung et al. 2012, St. Paul et al. 2014), single stranded RNA (TLR7 and 8) (Stewart et al. 2012, Sachan et al. 2015) and unmethylated CpG DNA (TLR 21) (Mallick et al. 2011) have been reported to be used as adjuvants in poultry vaccines, increasing significantly innate and adaptive immune responses. In this thesis, **in chapter 3** we investigated the role of CpG (TLR 21 agonists) as adjuvant of the H9N2 inactivated whole virus (IWV) vaccine in chickens. We found that the combination of several copies of CpG-A ODN and CpG-B ODN together in a T vector significantly up-regulated mRNA expression of cytokinesis including chicken-interferon-α (ch-IFN-α), chicken-interferon-β (ch-IFN-β), chicken-

interferon-γ (ch-IFN-γ) and chicken-interleukin-12 protein 40 (ch-IL-12p40) *in vitro* and *in vivo*. These cytokines both directly and indirectly promote adaptive immunity via enhancing the capacity of B lymphocytes to secrete antigen-specific antibodies.

6.2.2 Avian adaptive immune response

The adaptive immune response including the humoral and cellular immune response constitutes the second line of defense against pathogens. Lymphocytes including B cells and T cells are mainly responsible for the adaptive immune response and can specifically recognize antigens of pathogens.

6.2.2.1 Humoral immune response

In the case of the humoral immune response in chicken, for instance, activated B lymphocytes which initially develop in the bursa of avian Fabricius produce and secrete antigen-specific antibodies protecting the host from these pathogens. Sitaras et al (Sitaras et al. 2016a, Sitaras et al. 2016b) show that when there are sufficiently high antibodies titers (hemagglutination inhibition titers $\geq 2^3$) against challenge strain in over 86.5% population of vaccinated chickens, the transmission of HPAI H5N1 will be stopped regardless of the antigenic distance. Therefore, high titers of antibodies against AIV, estimated by hemagglutination inhibition (HI) assay and correlated with protection against transmission, may be very important for preventing transmission of AIV in the field. However, vaccines always fail to induce high HI titers against H9N2 AIV in poultry in the field (Bahari et al. 2015, Gu et al. 2017, Peacock et al. 2019). **In chapter 2**, we found that in 1-day-old commercial broilers, high HI titers of H9N2-specific antibodies were measured already at day 0 before vaccination, which we attributed to the presence of MDAs. After vaccination, the titers of vaccinated broilers decreased over time consistent with the degradation of MDAs. In 21-day-old commercial broilers, however, low HI titers ($< 2^3$) were detected at day 0 before vaccination, but HI titers significantly increased after vaccination. When analyzing the humoral immune responses of SPF chickens (no MDAs), they all mounted high HI titers after vaccination. These results indicated that MDAs may interfere with the vaccine efficacy in poultry. Furthermore, the results were confirmed in SPF chickens by using passively transferred antibodies (PTAs) to mimic MDAs in laboratory. Hence, these results strongly support our hypothesis that high titers of MDAs interfere with the humoral immune responses of chickens to vaccination.

Because of MDAs interference as well as the supposed importance of antibodies against AIV, it is essential to create new vaccines to induce high titers of H9N2-specific antibodies in the presence of MDAs in chickens. **In chapter 3**, we created a new CpG ODN plasmid-based adjuvant for the conventional H9N2 IWV vaccine. We

found that CpG ODN plasmid-based vaccine not only stimulated the innate but also adaptive immune responses even in the presence of PTAs used to mimic MDAs. The results indicated that the CpG ODN plasmid-based adjuvant had a great potential to overcome MDAs interference in poultry. Similar results can be found in mammals. When TLR-3 agonists (poly IC) and TLR-9 (functional homologue of TLR-21 of chickens) agonists (CpG ODN) are used together as the adjuvant of inactivated measles vaccines in cotton rats, high titers of measles virus (MeV)-specific antibodies (both neutralizing and non-neutralizing antibodies) can be generated even in presence of MeV-specific MDAs of cotton rats after vaccination (Kim and Niewiesk 2013).

As an alternative strategy to induce high titers of H9N2-specific antibodies in the presence of MDAs in chickens, using turkey herpesvirus (HVT) as a basis of a vector vaccine also have great potential. The cell-associated nature, the nature of the replication of the HVT vector, and the lack of expression of target antigens on the surface of infected cells or by the recombinant HVT vaccine probably all contribute to avoiding the MDAs interference against the vector and/or target pathogens, stimulating high titers of antibodies in the presence of MDAs (Bublot et al. 2007, Faulkner et al. 2013, Bertran et al. 2018, Le Gros et al. 2009). Therefore, **in chapter 4**, HVT was used as a vaccine vector to express H9 HA protein. The recombinant viral vector vaccine, rHVT-H9, could stimulate high titers of H9-specific antibodies in the presence of PTAs in chickens. These results suggest that the two new vaccines (inactivated with adjuvant and HVT vector) are both able to overcome MDAs interference inducing high humoral immune responses in poultry.

6.2.2.2 Cellular immune response

The cellular immune response involves T cells which are developed in the thymus. T cells comprise different cell types such as CD4 (cluster of differentiation 4) and CD8 (cluster of differentiation 8) T cells. The activated $CD4^+$ T cells will differentiate into a variety of T helper (Th) cell subsets which secrete cytokines facilitating the cellular and humoral immune responses (Okoye and Wilson 2011, Belz et al. 2002, Zhu and Paul 2010). Activated $CD8^+$ cells will proliferate and differentiate into cytotoxic T lymphocytes (CTLs) (Ho et al. 2011). CTLs, with the help of $CD4^+$ cells, can migrate to the infected site and destroy virus-infected cells resulting in the elimination of invading pathogens (Nakanishi et al. 2009).

T cell-mediated immunity (CMI) is considered to be important against respiratory viruses such as influenza viruses (Kapczynski et al. 2011, Seo and Webster 2001). Not only does the CpG ODN-based adjuvant enhance humoral immunity, but also significantly enhances CMI (Hartmann, Krieg and Erinrt 1999). **In chapter 3**, the addition of the CpG ODN plasmid-based adjuvant for the H9N2 IWV vaccine significantly increased the mRNA expression of cytokines such as chicken-interferon-

α (ch-IFN-α), chicken-interferon-β (ch-IFN-β), chicken-interferon-γ (ch-IFN-γ) and chicken-interleukin-12 protein 40 (ch-IL-12p40) in the presence of PTAs in chickens, which indicated good CMI as well. **In chapter 4**, using ELISpot and q-PCR, we also found potent CMI in the presence of PTAs in chickens stimulated by recombinant viral vector vaccine rHVT-H9.

6.2.2.3 Mucosal immune response

The mucosal immune system plays a very crucial role in controlling infection with influenza viruses in the upper respiratory tract (URP) of animals (Gould et al. 2017). In URP, activated B cells will mainly produce and secrete polymeric IgA isotype antibodies (Russell 1993). The secretory IgA (S-IgA) on the surface of the respiratory mucosal can neutralize infected viruses, eliminating viruses from the host. Compared to IgG, S-IgA plays a key role in preventing infection of influenza viruses (Suzuki et al. 2015). **In chapter 4**, the recombination rHVT-H9 vaccine induced higher S-IgA antibodies in lavage liquids from the nasal cavity and trachea compared to naïve SPF chickens. However, there was no big difference in S-IgA titers among the rHVT-H9, H9N2 IWV vaccine and PBS inoculated chickens with PTAs. These results suggest that rHVT-H9 may not stimulate potent mucosal immune responses in the presence of MDAs in poultry.

6.3 Mechanisms of MDAs interference

The mechanisms of MDAs interference with immune responses are still not clear. That is even the case for mammals where this is studied more. Generally, five main hypotheses have been proposed for mammals: (1) antigen neutralization (Albrecht et al. 1977) (2) epitope masking (Bergstrom, Xu and Heyman 2017, Brüggemann and Rajewsky 1982, Heyman and Wigzell 1984) (3) inhibition by Fcγ receptor IIB (FcγRIIB)-mediated signaling (Kim et al. 2011, Edwards 2015) (4) clearance of MDA-coated vaccine antigens (Siegrist 2003) (5) shaping the early-life B cells repertoire (Vono et al. 2019). In contrast, little information can be found about the mechanisms of MDAs interference in birds.

In chapter 5, Our data showed that only the antigen-binding portion, offered by H9N2-specific IgG ($F(ab)_2$), or only the Fc portion, offered by NDV-specific IgY (Fc), were not sufficient to interfere with the immune responses triggered by the H9N2 IWV vaccine. Instead, both the antigen-binding as well as Fc portion of autologous antibodies, offered by H9N2-specific IgY ($F(ab)_2$ +Fc), were required and responsible for reduced vaccine efficacy **(Fig. 1)**. The results are consistent with the inhibition by Fcγ receptor IIB (FcγRIIB)-mediated signaling hypothesis in mammals mentioned above.

Furthermore, Kim et al (Kim and Niewiesk 2013) proved the function of type I IFNs in overcoming MDAs interference in mammals. To be specific, they report that Poly I:C combined with CpG ODN helps the MeV-inactivated vaccine overcome MDAs interference. They attributed this success to the high level of type I IFNs stimulated by the combination of Poly I:C with CpG ODN. However, nobody is directly using type I IFNs to study whether type I IFNs can help overcome MDAs interference. **In chapter 5**, intravenous injection of recombinant type I chicken interferons (ch-IFNs), but not the intramuscular injection of plasmid encoding ch-IFNs, into chickens with PTAs, elicited potent immune responses after vaccination. The results directly proved that type I ch-IFNs could help the H9N2 IWV vaccine overcome MDAs interference in chickens **(Fig. 1)**.

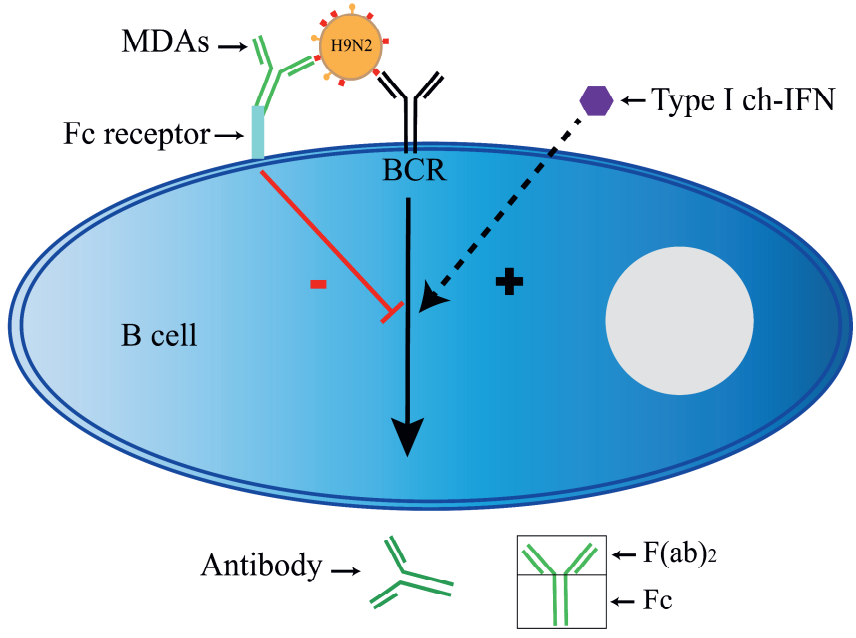

Figure 1. The mechanisms of MDAs interference to H9N2 vaccination.

In addition, **in chapter 5,** flow cytometry analysis showed that the percentage of $CD4^+$ and $CD8^+$ T cells were not affected during the whole experiment. These findings are similar to the general acknowledgment regarding MDAs interference in mammals that MDAs interfere with B cell activation, and thus lead to failing antibody generation, while T cells are generally not affected by MDAs (Karlsson et al. 1999, Brinc et al. 2007, Getahun and Heyman 2009, Orije et al. 2020). Unfortunately, our study failed to calculate the change of B cells. However, a recent study shows that MDAs do not hinder B cell activation but interfere with B cell differentiation into plasma cells, and T follicular helper (Th) cells prematurely decline in the presence of MDAs in cotton rats (Vono et al. 2019).

6.4 New measurements for assessing transmission of H9N2 AIV

When assessing the efficacy of vaccines in the control of infectious diseases, and especially zoonotic diseases, the ability to control the spread of viruses at the population level should be more important than only clinical protection and individual infection. However, most research about H9N2 AIV only focused on clinical protection and individual infection rather than virus transmission, possibly leading to constantly new infections without these being observed, also known as “silent spread”. Over time, the continued transmission of H9N2 AIV among animals may lead to the next influenza pandemic either directly crossing the species barrier or through the donation of internal genes to a pandemic virus (Caceres, Rajao and Perez 2021, RahimiRad et al. 2016, Peacock et al. 2019). Therefore, it is important to develop some new vaccines to stop transmission of H9N2 AIV in the field.

In chapter 4, we created a new live viral vector vaccine using turkey herpesvirus to express H9N2 HA protein. To assess the vaccine efficacy against transmission of homogeneous H9N2 AIV in chickens that received PTAs to mimic MDAs, we designed a transmission experiment and analyzed it by the stochastic S (susceptible)-I (infectious)-R (recovered) model. In this model, we estimated the reproduction ratio (R) value which is the average number of secondary infections caused by one typical infected animal (Diekmann, Heesterbeek and Metz 1990). The R value of rHVT-H9 vaccinated chickens were 1.75 (0.71 - 5.51) much smaller than that of the H9N2 IWV vaccine (12.76 (4.42 - 37.88)) and PBS ((0.85 - ∞) based on final size) inoculated chickens. These results indicated that the rHVT-H9 vaccine indeed reduced transmission of H9N2 AIV in poultry. For stopping transmission of pathogens, the R value should be below 1. However, the R value was still over 1 in rHVT-H9 inoculated chickens, although these inoculated chickens got significantly high antibody titers (HI $>2^6$) against H9N2 AIV. These results suggest that in addition to antibodies, some new measurements should also be used to assess the efficacy of vaccines, especially

against transmission of H9N2 AIV in poultry. For that, we might also want to look at other measurements of immune responses.

The mucosal immune response may be a more appropriate measurement to assess the efficacy of vaccines against transmission of H9N2 AIV. For highly pathogenic avian influenza viruses (HPAIV) such as H5N1, high antibodies titers (HI $>2^3$) against the challenge strain in over 86.5% population of vaccinated chickens are enough to stop transmission of the virus in SPF chickens (Sitaras et al. 2016a, Sitaras et al. 2016b). However, for low pathogenicity avian influenza virus (LPAIV) such as H9N2, high antibody titers fail to stop its transmission in SPF chickens (Cui et al. 2021). **In chapter 4**, our results also showed that H9N2 AIV is still transmitted in chickens that received PTAs, even if the rHVT-H9 vaccine stimulated high antibody titers against H9N2 AIV. The different outcomes may be because of the different virus-host interactions of HPAIV and LPAIV. For instance, HPAIV replicates systemically in chickens, while LPAIV mainly replicates in the respiratory and gastrointestinal tracts (Peacock et al. 2019). Hence, local mucosal immunity in respiratory and gastrointestinal tracts may play a more important role than systemically humoral immunity against transmission of H9N2 AIV. In contrast to IgG which only reduces viral pneumonia, IgA generated by mucosal immunity exerts a key role in preventing infection of human upper respiratory tract (UTR) mucosa with influenza viruses (Suzuki et al. 2015). Overall, we assume that the levels of local mucosal immunity represented by IgA in respiratory rather than systemically humoral immunity represented by IgY may be a new measurement for vaccines when assessing its efficacy in the control of H9N2 AIV transmission in poultry.

6.5 Strategies for vaccines based on the new measurement

6.5.1 Live attenuated vaccine

Live attenuated vaccine is an ideal influenza vaccine that is expected to induce mucosal immunity represented by IgA antibodies in the upper respiratory tract. In addition, the intranasal administration of live attenuated influenza vaccines (LAIVs) mimicking natural infection provides better cross-protection compared to inactivated vaccines. So far, a cold-adapt influenza virus has been used as LAIV for many years in humans and is licensed in the United States in 2003 (Maassab and Bryant 1999, Ambrose, Levin and Belshe 2011). This LAIV replicates well at a low temperature around 25 ℃ rather than at 37 ℃, stimulating local mucosal immune responses in the upper airway such as the nasal cavity, and deterring their replication in the lower airway including lung tissue. Several researchers have created some live attenuated H9N2 influenza vaccine candidates through the cold-adapted strategy (Wei et al. 2016) or NS1 gene truncations strategy (Chen et al. 2017, Chen et al. 2020). These LAIVs

against H9N2 AIV induce high titers of IgA, IgY and T cell immune responses in SPF chickens. However, because of the zoonotic potential of AIVs and the risk for virus reassortment, vaccination using LAIVs against H9N2 AIV is not desirable in poultry.

6.5.2 Inactivated vaccine

Intramuscularly or subcutaneously applied H9N2 inactivated vaccine mainly triggers humoral, rather than cellular and mucosal immune responses. Furthermore, intranasally applied inactivated vaccine is poorly immunogenic (Hagenaars et al. 2008, Worrall and Priadi 2009, De Geus et al. 2011). Therefore, we will discuss some new strategies that can be used to improve the efficacy of H9N2 inactivated vaccine in stimulating mucosal immunity in chickens.

Mucosal adjuvants can significantly increase the immunogenicity of inactivated vaccines and are important for inactivated vaccines to stimulate high local mucosal immune responses. A number of mucosal adjuvants have been widely reported, including enterotoxin (cholera toxin and heat-labile enterotoxin), toll-like receptors (TLR) ligands (CpG, Poly I:C, lipopolysaccharide, flagellin), mucoadhesives (Chitosan, Lectins), cytokines (chicken interleukin-1 beta, IFN-λ), etc (reviewed in (Wang, Wei and Liu 2020)). Most of these mucosal adjuvants can help inactivated vaccines stimulate strong IgA, IgY (IgG) and T cell responses through the nasal and/or oral delivery route.

To stimulate mucosal immunity by inactivated vaccines, the way we used to inactivate viruses also plays a key role. Conventionally, for making the H9N2 IWV vaccine, H9N2 AIV is generally inactivated with formalin or β-propiolactone, which are poor in cellular and mucosal immune responses. However, recently a gamma-irradiated inactivated vaccine against fowl cholera enhances strong mucosal immunity after intranasal vaccination in chickens (Dessalegn et al. 2021). This strategy may be also practical in the H9N2 IWV vaccine.

6.5.3 Live vector vaccine

Like LAIVs, many live vector vaccines are good vaccine candidates to stimulate potent IgA, IgY, and T cells immune responses in chickens such as Newcastle disease virus (NDV) (Rauw et al. 2009), Fowlpox virus (Townsend et al. 2017), and adenoviruses (Tutykhina et al. 2011). In addition, live vector vaccine against AIV is a good alternative approach to LAIVs since it has much more limited potential to be zoonotic and reassortment. However, these vector vaccines are themselves interfered with by vector-specific MDAs, which make them less effective in poultry, where all generations need to be vaccinated.

Some strategies for live vector vaccines are tested to overcome MDAs interference. For example, the modified NDV expressed the hemagglutinin-neuraminidase (HN) and fusion (F) genes from a different avian paramyxovirus, such as avian paramyxovirus type 8 (Steglich et al. 2013) or avian paramyxovirus type 2 (Kim, Paldurai and Samal 2017, Liu et al. 2018) can overcome NDV-specific MDAs interference. In addition, recombinant NDV-vectored experimental vaccine containing an antisense sequence of avian interleukin 4 (IL4R) and their backbones is able to overcome MDAs interference by administering *in ovo* at 18-day-old commercial eggs (Dimitrov et al. 2021). During the last few years, replication-defective human adenoviruses serotype-5 (hAdVs-5) has emerged as an attractive vector for the development of vaccines to overcome MDAs interference in poultry. In contrast to recombinant fowl adenovirus (FADVs), hAdVs-5 is not interfered with by anti-vector MDAs because the virus has shown no immunity in chickens (Romanutti, Keller and Zanetti 2020). Overall, when the live vector vaccines were not (or limited) interfered with by vector-specific MDAs, they may be excellent vaccine candidates against transmission of H9N2 AIV in poultry.

6.6 Conclusion

The work in this thesis showed that MDAs interfered with immune responses after inoculating with the H9N2 IWV vaccine in poultry, contributing to H9N2 vaccination failure in poultry (**chapter 2**). The H9N2 IWV vaccine with CpG ODN plasmid-based adjuvant could significantly stimulate humoral immune response and cytokinesis mRNA expression in the presence of PTAs used to mimic MDAs in SPF chickens (**chapter 3**). In addition, a recombinant turkey herpesvirus expressing H9N2 HA proteins (rHVT-H9) was designed, rescued, and identified. The rHVT-H9 stimulated strong humoral and cellular immunity in chickens with PTAs, reducing AIV shedding after challenge. Furthermore, although the rHVT-H9 reduced virus shedding, it did not sufficiently reduce the virus transmission in poultry since R value was still over 1(**chapter 4**). Finally, we explored the mechanisms of MDAs interference in chickens and found that only complete H9N2-specific MDAs interfered with immune responses rather than any other portions of antibody, and type I ch-IFNs helped the H9N2 IWV vaccine overcome MDAs interference. Furthermore, we confirmed that $CD4^+$ and $CD8^+$ T cells were not affected by MDAs in chickens (**chapter 5**). Taking all data together, I conclude that in addition to humoral and cellular immune responses, high levels of mucosal immune responses are also important to assess the efficacy of vaccines, especially in stopping transmission of H9N2 AIV in population.

6.7 Reference

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Summary

H9N2 avian influenza virus (AIV) is the most harmful and widespread low pathogenicity avian influenza virus (LPAIV) in the world. H9N2 AIV has a huge range of hosts. It is likely to mix or co-infect with other pathogens enhancing the severity of the clinical syndrome, higher rates of mortality in poultry and extending the period of H9N2 AIV shedding. Most importantly, H9N2 AIV can directly spill over from animals to humans or provide inner genes for other AIVs such as H5N1, H7N9, H5N6, H10N8 and H10N3 which also have been shown to infect humans. Therefore, some researchers are concerned that H9N2 AIV may be a spark to the emergence of the next influenza pandemic, either directly crossing the species barrier or through the donation of internal genes to a pandemic virus. Vaccination is the main and most effective way to control H9N2 AIV. However, H9N2 vaccination failure happens very often in poultry in practice, although these vaccines clam good efficacy in specific pathogen-free (SPF) chickens in laboratory. Therefore, my project was 1) to figure out what factors may interfere with the H9N2 vaccine efficacy to sufficiently reduce transmission in poultry, 2) to come up with some ideas to improve the vaccine efficacy, and 3) to explore the mechanisms that influence the vaccine efficacy.

In chapter 2, I figured out that H9N2-specific MDAs were one of reasons for H9N2 vaccination failure in poultry. I went to several poultry farms in China and did an epidemiological investigation for more than three months. I found that newly hatched broilers before vaccination had already got high antibodies against H9N2 AIV, which I interpreted as MDAs. Those newly hatched broilers with high titers of MDAs couldn't get potent immune responses after vaccination. Due to natural degradation of MDAs, 21-day-old broilers contained few MDAs, and these broilers got high immune responses after vaccination. All ages of SPF chickens got high humoral immune responses after vaccination. Therefore, I hypothesized that H9N2-specific MDAs interfered with immune response after inoculating with the H9N2 IWV vaccine in poultry. In order to test my hypothesis and reduce influence by other factors such antigenic distance between field strain and vaccine strain, coinfection with other pathogens in poultry, environment, chicken breed etc, I used passively transferred antibodies (PTAs) as a model to mimic MDAs in SPF chickens in laboratory. Using this model, I got the same results that MDAs hindered immune response after vaccination in SPF chickens. In addition, after challenge, chickens that received PTAs still shed virus after vaccination. On the other hand, chickens without PTAs after vaccination did not shed virus at all. Furthermore, I found that high and medium titers of MDAs may interfere with immune response, while low titers would not. These results in this chapter showed that MDAs interfere with immune responses after vaccinated with the H9N2 IWV vaccine.

In chapter 3, I developed a new adjuvant for the H9N2 IWV vaccine to overcome MDAs interference. I cloned different types of CpG ODN and their combination into

different plasmids. Firstly, I tested their immunomodulatory activities in chicken liver cell line (LMH). Then I found the combination of CpG-A and CpG-B based T vector plasmids (T-CpG-AB) showed the best immunomodulatory activities stimulating strong mRNA expression of cytokinesis including chicken-interferon-α (ch-IFN-α), chicken-interferon-β (ch-IFN-β) and chicken-interleukin-12 protein 40 (ch-IL-12p40). Therefore, T-CpG-AB plasmid was used as adjuvant to explore whether the plasmid could overcome MDAs interference and the minimum dose. Our results showed that 30 μg of T-CpG-AB plasmid-based adjuvant could significantly induce both strong humoral immune responses and cytokinesis expression even in the presence of PTAs in chickens. Our findings in this chapter suggest that T-CpG-AB plasmid can be an excellent adjuvant candidate for the H9N2 IWV vaccine to overcome MDAs interference in chickens.

In chapter 4, I developed a viral vector vaccine and assessed its efficacy in stopping transmission of H9N2 AIV among chickens that received high titers of PTAs (HI = 12 Log_2, 300 ml/chicken). Turkey herpesvirus (HVT) was used as a vaccine vector to express H9 hemagglutinin (HA) protein. I successful rescued the recombinant viral vector vaccine (rHVT-H9) in primary chicken embryonic fibroblasts (CEFs). Western blot and indirect immunofluorescence assay (IFA) showed that the rHVT-H9 consistently expressed HA proteins. In addition, the rHVT-H9 had similar growth kinetics to parent HVT. Preliminary animal experiments showed that the rHVT-H9, compared to conventional H9N2 IWV vaccines, stimulated a more robust humoral immunity in the presence of PTAs in chickens. In order to assess the efficacy of the rHVT-H9 vaccine in reducing transmission of H9N2 AIV in the presence of MDAs in chickens. I used PTAs to mimic MDAs and designed a transmission animal experiment. The transmission animal experiment showed that the rHVT-H9 induced both humoral and cellular immunity in the presence of PTAs. Furthermore**,** I collected oronasal and cloaca swabs samples after homologous challenge to detect virus shedding by inoculating PBS from these samples into 10-day-old SPF embryonated chicken eggs (ECEs) which were detected by hemagglutination (HA) assay. The results showed that the rHVT-H9 reduced the virus shedding period. Using SIR stochastic model, we found that the rHVT-H9 decreased the reproduction ratio (R) value in chickens that received PTAs, but not (yet) sufficiently (R > 1) In summary, in this chapter, we rescued the new rHVT-H9 vaccine, which stimulated strong humoral and cellular immunity even in the presence of PTAs in chickens comparable with the conventional H9N2 IWV vaccine, which suggests that the recombinant viral vector vaccine rHVT-H9 can reduce transmission of H9N2 AIV in poultry.

In chapter 5, I explored the mechanisms of MDAs interference. In order to identify which portion of MDAs interfere with immune response after inoculating with the H9N2 IWV vaccine, antibodies against different antigens and from different species

were used in this chapter. Homologous antibodies against H9N2 (H9N2-specific IgY), heterologous antibodies against H9N2 (H9N2-specific IgG) and homologous antibodies against Newcastle disease virus (NDV-specific IgY) were transferred intravenously into SPF chickens to mimic the complete MDAs ($F(ab)_2$ + Fc of H9N2-specific IgY), the antigen-binding portion of MDAs ($F(ab)_2$ of H9N2-specific IgG) and the Fc-binding portion of MDAs (Fc of NDV-specific). The results showed that only complete MDAs (H9N2-specific IgY) hindered immune responses. Using the PTAs (H9N2-specific IgY) model, our data showed that intravenous injection of recombinant chicken interferons (ch-IFNs), but not intramuscular injection of plasmid-encoded ch-IFNs, elicited potent immune responses after vaccination. To identify whether MDAs interfered with the activation of T cells, I performed a flow cytometry analysis. The results showed that the percentage of $CD4^+$ and $CD8^+$ T cells were not affected by PTAs (H9N2-specific IgY) during the whole experiment while. Our results in this chapter uncover some aspects of the mechanisms of MDAs interference to H9N2 vaccination in chickens.

In the last chapter, **chapter 6**, I discussed immune responses after vaccination, some new measurements to assess the efficacy of vaccines in stopping transmission (correlates of protection against transmission) of H9N2 AIV and future perspectives of vaccines in poultry based on new measurements. The immune system includes both innate and adaptive immunity. The two parts are highly integrated, and both are important to eliminate invasive pathogens. Therefore, an ideal vaccine should have the capacity to trigger both innate and adaptive immune responses at the same time in terms of immune responses. For inactivated vaccines, adjuvant can help them trigger potent innate immunity and adaptive immunity. For this, toll-like receptors (TLRs) agonist-based adjuvants hold great promise as vaccine adjuvant candidates in chickens. Live vaccines are also good candidates to stimulate both innate and adaptive immunity. However, still some problems need to be solved with live vaccines. In order to stop transmission of H9N2 AIV or other respiratory viruses, I believe mucosal vaccines may be a good choice, and therefore in this last chapter, I provided some ideas for mucosal vaccines in the end.

Overall, this thesis provides a deeper understanding of H9N2 vaccination failure in poultry and sparks some ideas to stop transmission of respiratory viruses.

Appendices

Curriculum vitae

About the author

Xue Pan was born on the 25th of November 1991 in Xidai village, a small and remote village in the middle of China. When he was young, he used to feed cattle on mountains, feed swine and chickens in the field. Therefore, he is very interested in nature and animals.

Because of his interests, Xue Pan chose to major in Animal medicine in Science and Technology of Henan University in Luoyang City in China. He completed his BSc in September 2015 with the thesis titled "Tributyltin affects anxiety and bunching behavior of Gobiocypris Rarus". At the same year, he registered as a master student in Shanghai Veterinary Research Institute (SHVRI) of Chinese Academy of Agricultural Sciences, majoring in Veterinary Medicine and researching in avian influenza viruses. After three years study, he achieved his MSc degree with the thesis titled "The molecular basis of avian-dog interspecies transmission of H3N2 avian-origin canine influenza virus". In order to further his study, Xue Pan enrolled for a sandwich PhD student in Quantitative Veterinary Epidemiology at Wageningen University & Research (WUR) in the Netherlands, supervised by Prof. Zejun Li (SHVRI) and Prof. Mart de Jong, Dr Nancy Beerens, and Maria Forlenza (WUR). At the beginning of his PhD project, he spent more than three months in the field to figure out the reasons for H9N2 vaccination failure in poultry supervised by Dr Haitao Zhang in Jiangsu Lihua Animal Husbandry in China. After almost five years study, he finally completed his PhD project with the PhD thesis titled "Development of immune response against H9N2 avian influenza after vaccination". The main results of his PhD research are presented in this thesis.

Pree-reviewed publications

Pan, X., X. Su, P. Ding, J. Zhao, H. Cui, D. Yan, Q. Teng, X. Li, N. Beerens & H. Zhang (2022) Maternal-derived antibodies hinder the antibody response to H9N2 AIV inactivated vaccine in the field. *Animal Diseases,* 2**,** 1-9.

PAN Xue, CHEN Xin-wu, JI Ya-ning, LI Xue-song, LIU Qin-fang, CHEN Hong-jun, YANG Jian-mei,TENG Qiao-yang, LI Ze-jun. (2019) Characterization of virus-like particle vaccine against H9N2 avian influenza virus. *Chinese Journal of Animal Infectious Diseases*, 27(1):1-7.

Xue Pan, Qinfang Liu, Shiqi Niu, Dongming Huang, Dawei Yan, Qiaoyang Teng, Xuesong Li, Nancy Beerens, Maria Forlenza, Mart C.M. de Jong and Zejun Li (2022) Efficacy of a recombinant turkey herpesvirus (H9) vaccine in preventing transmission of homologous H9N2 virus in chickens with maternal-derived antibodies. *Vaccine*. (under review).

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Su, X., Shuo, D., Luo, Y., **Pan, X**., Yan, D., Li, X., & Li, Z. (2022). An Emerging Duck Egg-Reducing Syndrome Caused by a Novel Picornavirus Containing Seven Putative 2A Peptides. *Viruses*, 14(5), 932.

Conference proceedings

Xue Pan, Qinfang Liu, Shiqi Niu, Dongming Huang, Dawei Yan, Qiaoyang Teng, Xuesong Li, Nancy Beerens, Maria Forlenza, Mart C.M. de Jong and Zejun Li (2022) Recombinant turkey herpesvirus influenza (H9) vaccine partially overcomes maternal antibodies interference in chickens. 16th Vaccine Congress, poster presentation (Riva del Garda, Italy).

Training and Supervision Plan (TSP)	Graduate School WIAS	
A. The Basic Package	year	credits *
WIAS Introduction Day (mandatory)	2019	0.3
Scientific Integrity & Ethics in Animal Sciences (mandatory)	2019	1.5
WIAS Course on Essential Skills (Frank Little) (recommended)	2019	1.2
Subtotal Basic Package		3
B. Disciplinary Competences	year	credits
Research proposal	2019	6.0
Management of Infections and Diseases in Animal Populations QVE	2019	6.0
Quantitative Discussion Group (QDG)	2019	1.0
Subtotal Disciplinary Competences		13
C. Professional Competences	year	credits
Effective Academic Development	2018	3.0
Mobilizing your scientific network	2019	1.0
Writing Grant Proposals	2019	2.0
Searching and Organising Literature	2019	0.6
Advanced Statistics course Design of Experiments	2022	0.8
Scientific Writing	2022	1.8
Scientific Publishing	2022	0.3
WIAS Course The Final Touch	2022	0.6
Career Perspectives	2022	1.6
Subtotal Professional Competences		12
D. Societal Relevance (recommended)	year	credits
Communication with the Media and the General Public	2022	1.0
WIAS magazine research highlight	2022	1.0
Subtotal Professional Competences		2
E. Presentation Skills (maximum 4 credits)	year	credits
title of presentation, name of conference/seminar, place, date, oral / poster		
16th Vaccine Conference, Riva del Garda Congress Centre, Italy (poster)	2022	1.0
Subtotal presentations		1
F. Teaching competences (max 6 credits)	year	credits
Supervising MSc in SHVRI, Shanghai, China	2019-2021	3.0
Subtotal Teaching competences		3
Education and Training Total (minimum 30 credits)*		34

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