



Maternal-derived antibodies hinder the antibody response to H9N2 AIV inactivated vaccine in the field

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Abstract

The H9N2 subtype avian influenza virus (AIV) inactivated vaccine has been used extensively in poultry farms, but it often fails to stimulate a sufficiently high immune response in poultry in the field, although it works well in laboratory experiments; hence, the virus still causes economic damage every year and poses a potential threat to public health. Based on surveillance data collected in the field, we found that broilers with high levels of maternal-derived antibodies (MDAs) against H9N2 virus did not produce high levels of antibodies after vaccination with a commercial H9N2 inactivated vaccine. In contrast, specific pathogen-free (SPF) chickens without MDAs responded efficiently to that vaccination. When MDAs were mimicked by administering passively transferred antibodies (PTAs) into SPF chickens in the laboratory, similar results were observed: H9N2-specific PTAs inhibited humoral immunity against the H9N2 inactivated vaccine, suggesting that H9N2-specific MDAs might hinder the generation of antibodies when H9N2 inactivated vaccine was used. After challenge with homologous H9N2 virus, the virus was detected in oropharyngeal swabs of the vaccinated and unvaccinated chickens with PTAs but not in the vaccinated chickens without PTAs, indicating that H9N2-specific MDAs were indeed one of the reasons for H9N2 inactivated vaccine failure in the field. When different titers of PTAs were used to mimic MDAs in SPF chickens, high (HI = 12 log₂) and medium (HI = log₂ 9) titers of PTAs reduced the generation of H9N2-specific antibodies after the first vaccination, but a booster dose would induce a high and faster humoral immune response even of PTA interference. This study strongly suggested that high or medium titers of MDAs might explain H9N2 inactivated vaccine failure in the field.

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

Introduction

H9N2 subtype avian influenza virus (AIV) is the most widespread and harmful low pathogenic avian influenza virus (LPAIV) in large parts of the world and

therefore poses an enormous threat to both the global poultry industry and human food security. In addition, H9N2 AIV might contribute to 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 and pigs (Trock et al. 2015; Peacock et al. 2019; Pusch and Suarez 2018; Song and Qin 2020).

Vaccination is the main strategy for controlling H9N2 AIV in poultry. H9N2 inactivated whole virus

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vaccine is currently the main vaccine used in many countries, such as China, Israel, South Korea, Morocco, Egypt, Pakistan, Egypt, and 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 collected from several poultry farms in China, broilers are normally vaccinated three times before being sold when they are approximately 45 days old to protect commercial broilers against H9N2 AIV: at 1-day-old with 0.1 mL of inactivated vaccine, 14-day-old with 0.2 mL of inactivated vaccine and 21-day-old with 0.3 mL of inactivated vaccine. However, most vaccination programs against H9N2 AIV have shown to be ineffective against infection and transmission in the field, although those vaccines have been reported to work well in the laboratory (Cui et al. 2021; Bahari et al. 2015; Gu et al. 2017; Peacock et al. 2019).

Unfortunately, researchers have not yet clearly determined what factors contribute most to H9N2 vaccination failure in the field. Antigenic drift between vaccine and field strains is considered as an important factor. Thus, updating vaccines constantly seems to be indispensable (Kapczynski and Swayne 2009; Capua and Cattoli 2013; Balish et al. 2010; Cattoli et al. 2011). The hemagglutination inhibition (HI) assay has been regarded as the most effective method to measure changes in antigenicity and has been used to assess the antigenic properties of AIVs for years (Donald and Isaacs 1954). Antigenic distance between vaccine and endemic strains has been evaluated by the differences in HI titers of, for example, the field virus against the field virus and of the same virus against the vaccine virus. The larger the discrepancies, the greater the antigenic distance will be. However, Sitaras et al. proved that when sufficiently high HI titers (over 3, i.e., $8 \log_2$) were present against the challenge strain in over 85% of vaccinated animals, the transmission of HPAIV H5 AIV was stopped, regardless of the antigenic distance (Sitaras et al. 2016a; Sitaras et al. 2016b). This result implies that for large antigenic differences, high HI titers against the vaccine virus are needed. Thus, a strong immune response and proper vaccination coverage would compensate for the antigenic distance and protect animals from infection and transmission (Swayne et al. 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).

Maternal-derived antibodies (MDAs) have been reported to interfere with the active immune response in many species and hinder the efficacy of most types of vaccines, such as inactivated vaccines, vector vaccines, subunit vaccines and live attenuated vaccines in mammals (Niewiesk 2014; Bahgat et al. 2009; Faulkner et al. 2013; Maas et al. 2011). However, researchers have not

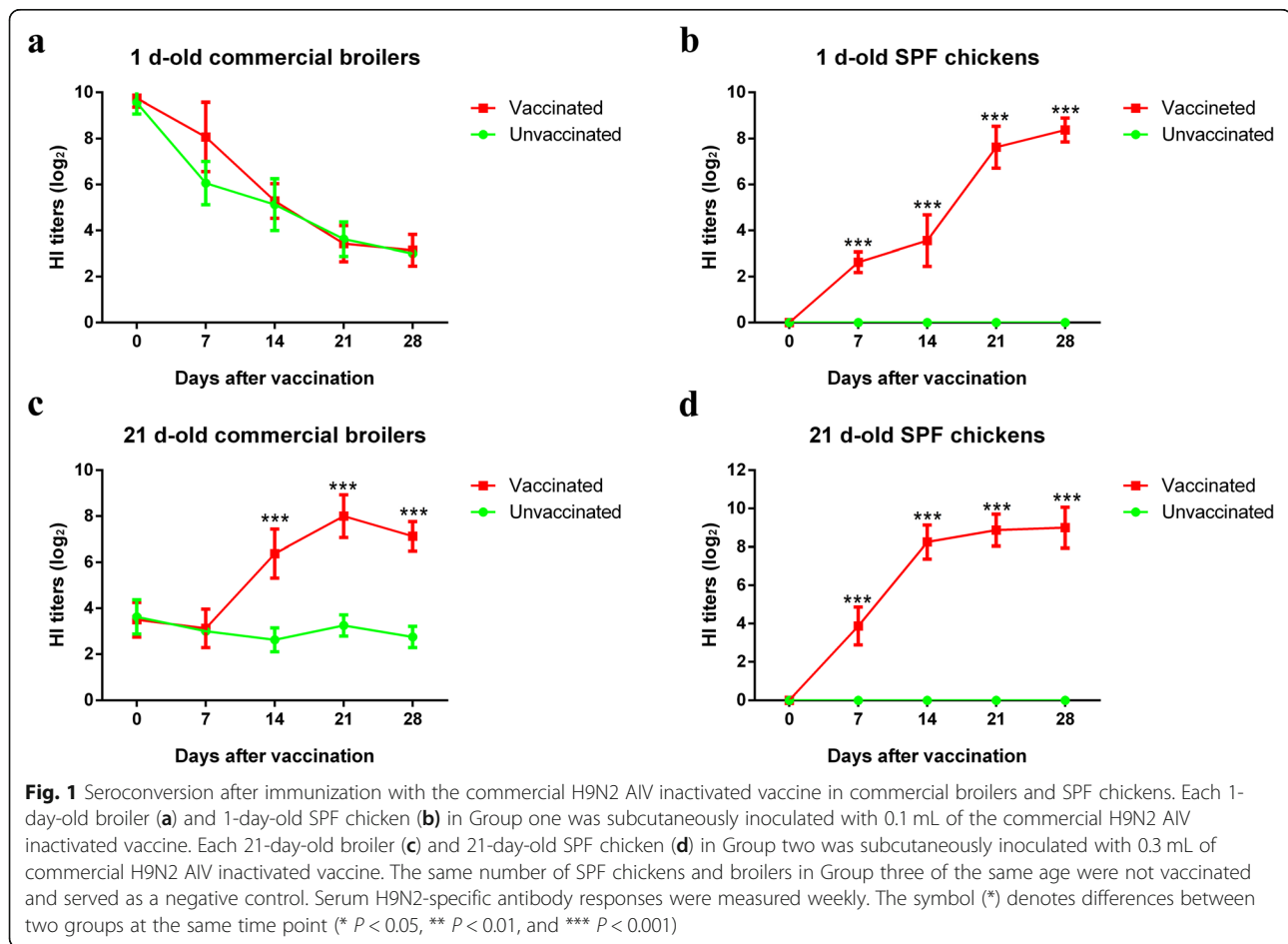
clearly identified whether MDAs are one of the reasons for H9N2 inactivated vaccine failure. In the present study, the dynamics of MDAs in poultry and the effect of MDAs on the immune response after inoculation with an inactivated H9N2 vaccine were explored.

The final MDAs transferred from dams have a high degree of variability in individual broilers (Gharaibeh et al. 2008), and thus MDAs are difficult to study in the field. However, the hyperimmune serum mainly contains IgY. This antibody has similar isotype proportions to MDAs and therefore has been used to mimic MDAs (Hamal et al. 2006; Forrest et al. 2013). In the present study, passively transferred antibodies (PTAs) were used as a model in specific pathogen-free (SPF) chickens to mimic MDAs in the laboratory and to explore the possible reasons for H9N2 inactivated vaccine failure in poultry in the field.

Results

MDAs interfere with broilers' humoral immune response in the field

Commercial H9N2 inactivated vaccine has been used in poultry for more than 25 years in China (Jiang et al. 2012; Liu et al. 2020), and it has been proven to exhibit very good efficacy in SPF chickens in the laboratory, but H9N2 AIV is still prevalent among poultry farms. SPF chickens of the same age and commercial broilers were raised together and vaccinated to assess their immune responses and explore factors that may contribute to H9N2 inactivated vaccine failure in the field. The results are shown in Fig. 1. HI titers of 1-day-old commercial broilers were $9.6 \pm 0.5 \log_2$ at the moment of vaccination, which we interpreted to be attributed to MDAs, and the titers decreased gradually to approximately $3 \log_2$ at day 28 when animals were not vaccinated. After applying the commercial H9N2 inactivated vaccine to immunize the commercial 1-day-old broilers, HI titers also decreased gradually and were not different from those of the unvaccinated 1-day-old commercial broilers (Fig. 1a). In contrast, HI titers of 1-day-old SPF chickens were zero at the day of vaccination (interpreted as no MDAs present) and were significantly increased seven days after vaccination when inoculated with the commercial vaccine (Fig. 1b). HI titers of the commercial broilers after 21 days were low ($3.6 \pm 0.7 \log_2$), which were interpreted as a reduction in MDAs. Without vaccination, the titers remained low. On the other hand, HI titers of both commercial broilers and SPF chickens increased after vaccination when starting with 21-day-old chickens (i.e., with fewer remaining MDAs). HI titers of all vaccinated groups at 21-day-old days of age were significantly higher than those of the



corresponding unvaccinated groups (Fig. 1c, d). All these results indicated that high titers of MDAs in broilers at the moment of vaccination may explain H9N2 inactivated vaccine failure in the field.

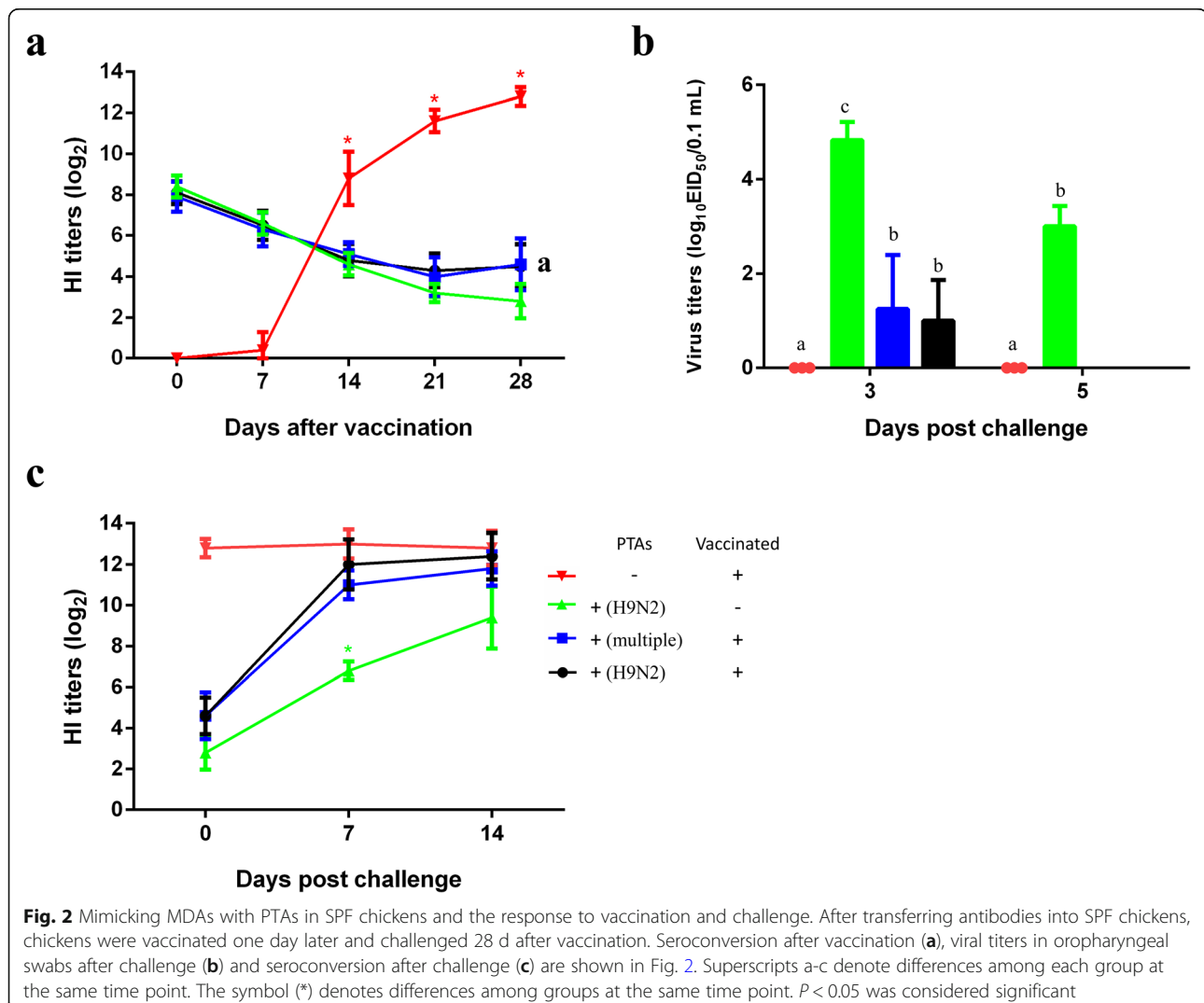
Mimicking MDAs with PTAs interferes with the humoral immune response of SPF chickens in laboratory

PTAs were used to mimic MDAs in laboratory and identify whether MDAs are indeed one of factors contributing to H9N2 inactivated vaccine failure in the field. 1-day-old SPF chickens were passively transferred with 0.3 mL of high titers ($\text{HI} = 12 \log_2$) of H9N2-specific antibody and then vaccinated to show their immune response. As shown in Fig. 2a, after passive transfer, HI titers of those chickens were approximately $8 \log_2$, which was similar to that of 1-day-old commercial broilers in the field (Fig. 1a). The dynamics of HI titers of PTAs in SPF chickens were also similar to those of MDAs in commercial broilers and ranged from $8.4 \pm 0.5 \log_2$ to $2.8 \pm 0.8 \log_2$. Two different hyperimmune sera were used to mimic MDAs in this study: one was collected from commercial hens in the field that were vaccinated

with multiple types of vaccines, including H9N2, H5N1 and H7N9 inactivated vaccines, live attenuated Newcastle disease virus (NDV) vaccine, and VAXXITEK (herpesvirus of turkeys + infectious bursal disease) vector vaccine; another was collected from SPF chickens in laboratory, which contained antibodies against only H9N2 AIV. Figure 2a shows that both types of PTAs hindered the generation of H9N2-specific antibodies after vaccination. In contrast, HI titers of SPF chickens without PTAs increased gradually after vaccination and were significantly higher than those of SPF chickens with PTAs 14 days after vaccination.

Viral shedding and antibody titers after H9N2 challenge

MDAs clearly interfered with chickens' humoral immune responses. Chickens were challenged 28 days after vaccination and viral shedding was detected to obtain insights into the interference induced by MDAs. As shown in Fig. 2b, viral titers in the oropharynx of unvaccinated group were the highest at $4.9 \pm 0.4 \log_2$ at 3 days post-challenge (dpc), and viral titers were 1.5 ± 0.9 and $1.2 \pm 0.7 \log_2$ in chickens with PTAs from commercial hens and SPF



chickens, respectively. On the other hand, no viral shedding was detected in the vaccinated group without PTAs. At 5 dpc, the virus was detected only in oropharyngeal samples from unvaccinated group ($EID_{50} = 3.1 \pm 0.4 \log_2/0.1 \text{ mL}$). All detected viruses were in oropharyngeal swabs.

Compared with unvaccinated chickens, vaccinated chickens were more likely to have a higher and faster antibody response after challenge. The antibody titers of chickens vaccinated with PTAs from commercial hens ranged from 4.6 ± 1.1 to $11 \pm 0.7 \log_2$; the antibody titers of chickens vaccinated with PTAs from SPF chickens ranged from 4.6 ± 0.9 to $12 \pm 1.2 \log_2$ at 7 dpc (Fig. 2c).

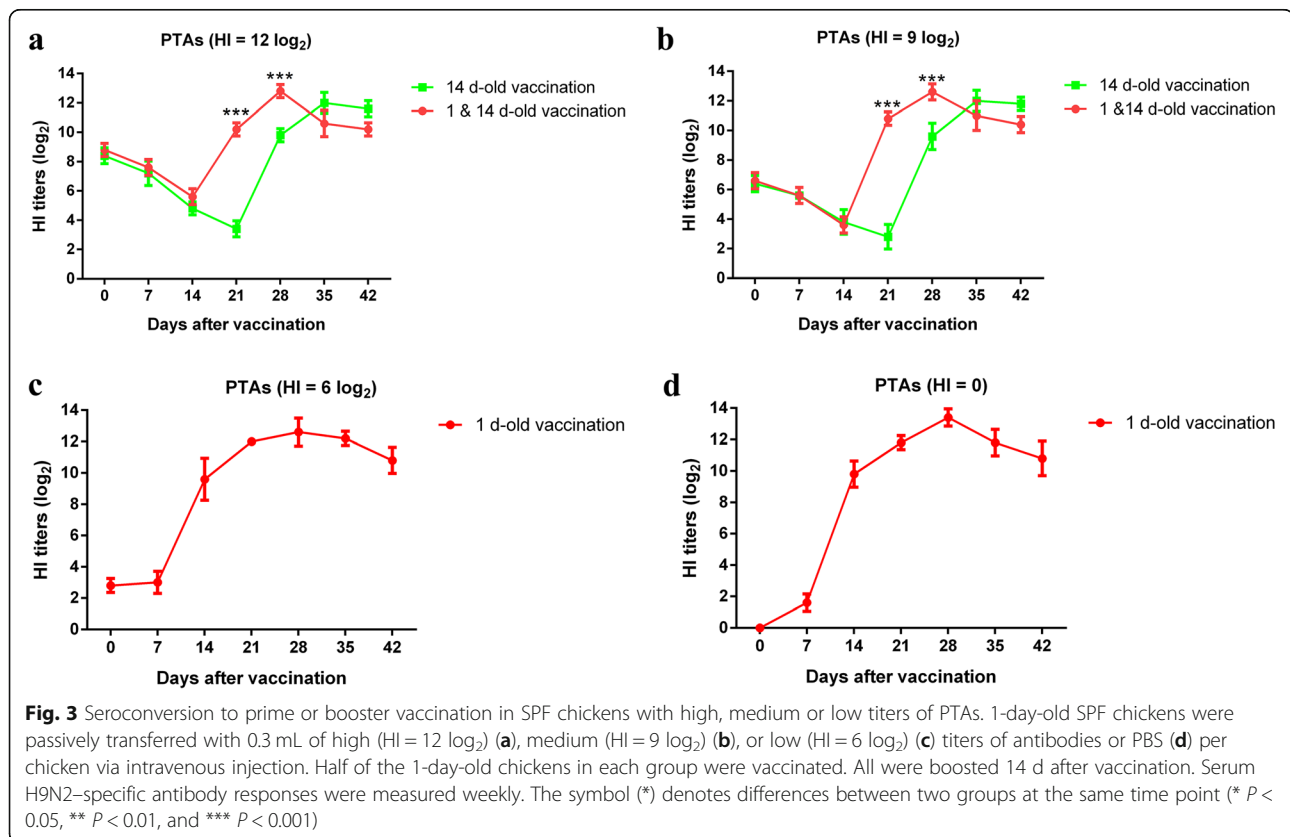
High and medium titers of PTAs interfered with chickens' humoral immune responses after the first vaccination

High ($HI = 12 \log_2$), medium ($HI = 9 \log_2$), and low ($HI = 6 \log_2$) titers of H9-specific antibodies and phosphate-buffered saline (PBS) were used as PTAs in the present study to obtain a better understanding of

the degree of interference induced by MDAs. High (Fig. 3a) and medium (Fig. 3b) titers of PTAs indeed attenuated the development of antibodies after the first vaccination, while low (Fig. 3c) titers of PTAs and PBS did not. However, after the booster vaccination, the antibody titers of chickens injected with both high and medium titers of PTAs increased immediately from 5.6 ± 0.5 to $10.2 \pm 0.4 \log_2$ and 3.6 ± 0.5 to $10.8 \pm 0.4 \log_2$, respectively, one week later, which were similar to the antibody levels of vaccinated SPF chickens without PTAs.

Discussion

H9N2 AIV has caused substantial damage to economies and public health. The inactivated vaccine has been used regularly in the field in several countries to control it, but the vaccine fails to stop the transmission and infection of H9N2 AIV in poultry in the field. In the present study, we found that MDAs might explain H9N2



vaccination failure in poultry in the field. A study conducted in the field and laboratory showed that H9N2-specific MDAs indeed interfered with the immune response after vaccination with the inactivated H9N2 vaccine in chickens. Furthermore, vaccination of chickens without PTAs inhibited viral shedding through the oropharyngeal route but not in chickens vaccinated with PTAs at 3 days after challenge. In addition, high and medium titers of PTAs interfered with the humoral immune response in chickens after vaccination. However, a booster dose induced a higher and faster humoral immune response, even under the condition of PTA interference.

H9N2 inactivated vaccine shows perfect efficacy in SPF chickens in the laboratory, but it often fails in commercial broilers in the field (Kim et al. 2010). Several possible explanations have been proposed. Antigenic drift is generally presumed to be a major reason. Mixed infection or concurrent infection of H9N2 vaccination with other pathogens may also contribute to vaccination failure. Moreover, other factors, such as improper vaccination procedures, incorrect use of vaccines, density, condition and species of chickens, may also contribute to this phenomenon. However, the contribution of MDAs to H9N2 inactivated vaccine failure has received less attention. In this research, SPF chickens were

vaccinated with inactivated H9N2 (H514) homologous to the virus challenge in laboratory to minimize the effects of other factors on vaccine efficacy. All experiments were conducted in the Biological Safety Level 2 (BSL2) facility. Using a PTA model to mimic MDAs in SPF chickens, this study showed that PTAs interfered with chickens' immune response to the H9N2 inactivated vaccine, indicating that MDAs were also one of the factors contributing to H9N2 inactivated vaccine failure in poultry in the field. Similar results were reported by Forrest et al. (Forrest et al. 2013), they used two different sources of antibodies (from field-vaccinated layer chickens or vaccinated SPF chickens) to mimic H5N2-specific MDAs in three-week-old SPF chickens in different ways. Similar to the present study, all sources of PTAs reduced the generation of antibodies against H5N2 AIV. MDAs are also considered a factor contributing to H5N1 inactivated vaccine failure in broilers in Egypt (Abdelwhab et al. 2012). The decrease in vaccine immune efficiency caused by MDA interference may further affect the prevention and control of AIV in poultry in the field (Kim et al. 2010; Maas et al. 2011).

MDAs protect offspring at a young age from many infectious diseases at the beginning of their lives when they are vulnerable because of the immature immune system (Forrest et al. 2013; Maas et al. 2011), but MDAs

do not protect chickens against H9N2 AIV during their whole life since their levels decrease rapidly with age. In this paper, HI titers of 1-day-old chickens with PTAs or MDAs gradually decreased from approximately $9 \log_2$ to approximately $3 \log_2$ in 21-day-old chickens with or without vaccination, and thus they were not protective against H9N2 AIV. In contrast, after challenge, the virus was not detected in vaccinated chickens without PTAs, as HI titers of those chickens were approximately $12 \log_2$, which protected chickens from H9N2 AIV (Fig. 2b). Similar results were obtained by other researchers (Chen et al. 2017; Kim et al. 2021; Zhao et al. 2017). A potential explanation for this result is that HI titers must be over $5 \log_2$ to protect chickens from H9N2 AIV in the field.

Understanding the mechanism of MDAs interference is essential for developing next-generation vaccines to overcome/bypass MDAs interference. In mammals, MDAs inhibit all immune cells that react specifically to antigens and antibodies, including germinal center B cells, plasma cells (PCs) and memory B cells (MBCs) (Bergstrom et al. 2017; De Vriese et al. 2010). However, Maria Vono reported that only high levels of MDAs block the generation of both antibodies and MBCs, while low titers of MDAs do not hinder the development of MBCs in mice (Vono et al. 2019). The difference between those studies is probably because different models and antigens were used. In avian species, researchers have not elucidated whether MDAs interfere with immune cells. The present study clearly showed that MDAs interfered with PCs, but a booster dose resulted in a higher and faster humoral immune response, even under the condition of MDA interference, indicating that MDAs may not interfere with memory B cells (MBCs) in chickens. Further research is needed to explore the mechanism by which MDAs interfere with vaccines in avian species in the future.

Based on the results from the present study, some suggestions for vaccination protocols to control H9N2 AIV in the field are provided. First, HI titers remained high after the booster dose was administered at 14 d (Fig. 3), therefore, we suggested that a third vaccination is unnecessary in 21-day-old broilers according to the vaccination procedure in poultry farms mentioned above. Second, vaccination of broilers in hatcheries is advised since the second shot on their 14th days might induce a higher and faster humoral immune response (Fig. 3). Next, although a booster dose potentially induced a high immune response in SPF chickens with PTAs in the laboratory, similar results may not be easily obtained in broilers in the field since several other factors may affect the vaccine efficacy mentioned above. Therefore, in addition to vaccination, a good long-term surveillance system, maintenance of environmental hygiene, and

training of poultry workers are also important. Finally, the traditional H9N2 inactivated vaccine used in the field requires updating with new vaccines that are able to overcome/bypass MDAs or are mixed with other types of vaccines from different generations.

Conclusions

Overall, this study suggested that H9N2-specific MDAs were one of the factors contributing to H9N2 inactivated vaccine failure in the field. High and medium titers of MDAs interfered with the humoral immune response in broilers, but a booster dose induced a higher and faster humoral immune response, even under the condition of MDA interference. Moreover, understanding the mechanism of MDA interference in avian species is essential to explore new methods to tackle this problem in the future.

Methods

Animals and viruses

The field experiment was conducted in the Biotechnology Research Laboratory, Jiangsu Lihua Animal Husbandry Co. Ltd. (JSLH), Changzhou 213,168, 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 (Beijing, China) and hatched in the laboratory of JULH. The laboratory experiment was performed in SHVRI. The 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 with a good environment and sufficient space, and birds had full access to feed and water.

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 10-day-old SPF embryonated chicken eggs (ECEs) (Beijing Merial Vital Laboratory Animal Technology Co., Ltd.).

Vaccine and hyperimmune serum preparation

The commercial H9N2 (SS strain) water-in-oil inactivated vaccine (Guangdong Wens Dahuanong Biotechnology Co., Ltd.) is generally used in the field. In laboratory experiments, H9N2 AIV (H514) ($10^{9.25}$ EID₅₀/0.1 mL) was inactivated with 1:2000 β -propiolactone (BPL) under constant 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 into three eggs and incubated for 48 h to confirm the loss of infectivity by performing a hemagglutination (HA) assay. Based on the phylogenetic analysis, the 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 (0.85 g/cm³) adjuvant (Lone et al. 2017) at a volume ratio of 3:7 according to the instructions.

Hyperimmune serum was collected from commercial hens housed at poultry farms and contains IgY against multiple antigens. The VG71 BPL-inactivated H514 vaccine was used to generate hyperimmune serum in SPF chickens by vaccinating chickens three times, once every two weeks in the laboratory. This hyperimmune serum contains IgY only against H9N2 AIV.

Experimental design

Experiment one

The effect of MDAs on the efficacy of a commercial H9N2 inactivated vaccine in the field was analyzed. Three groups of chickens were used. Each group consisted of 16 1-day-old chickens consisting of half commercial broilers and half SPF chickens, and one group was housed in one isolator. In Group 1, all chickens were vaccinated subcutaneously in the neck with the commercial H9N2 inactivated vaccine at a dose of 0.1 mL per chicken at day one after hatching; in Group 2, all chickens were immunized with 0.3 mL per chicken of the same vaccine at 21-day-old days of age according to the company's vaccination procedure in the field. In Group 3, chickens were not vaccinated and served as a negative control. Serum samples were collected from each chicken at 0, 7, 14, 21 and 28 dpv.

Experiment two

MDAs were mimicked with PTAs in SPF chickens in the laboratory. Four groups of chickens were used. A total of 0.3 mL of hyperimmune serum with HI titers of 12 log₂ against the H9N2 virus was administered by intravenous injection into 1-day-old SPF chickens. Chickens that received 0.3 mL of phosphate-buffered saline (PBS) as a vaccination served as a positive control (Group 1, *n* = 5). Chickens that received 0.3 mL of hyperimmune serum from SPF chickens without vaccination served as a negative control (Group 2, *n* = 5). Chickens also received hyperimmune serum from commercial poultry hens (Group 3, *n* = 5) or from SPF chickens (Group 4, *n* = 5). One day after administering PTAs, all chickens were vaccinated with 0.1 mL of VG71 BPL-inactivated H514 vaccine per chicken. Chickens in each group were challenged intravenously with 0.1 mL 10⁶ EID₅₀/0.1 mL of H514 at 28 dpv. Oropharyngeal and cloacal swabs were collected at 3 and 5 d postchallenge, respectively.

Serum samples were collected from each chicken at 0, 7, 14, 21, 28, 35 and 42 dpv.

Experiment three

The effects of different titers of H9N2-specific PTAs on the generation of antibodies was analyzed. For this experiment, 0.3 mL of four different titers of antibodies was transferred into 1-day-old SPF chickens (*n* = 10), and then half of these chickens from each group were vaccinated with 0.1 mL of VG71 BPL-inactivated H514 vaccine per chicken one day later (Group 1: HI = 12 log₂; Group 2: HI = 9 log₂; Group 3: HI = 6 log₂; Group 4: HI = 0 (PBS)). Then, all chickens were vaccinated with 0.2 mL of the H514 vaccine 14 d later according to the vaccination procedure in the field. Serum samples were collected from each chicken at 0, 7, 14, 21 and 28 days post vaccination (dpv).

Hemagglutination inhibition (HI) assay

The dynamics of antibodies were tested using HI assay as previously described (Suarez et al. 1998). HI titers were determined using the BPL-inactivated H514 virus. The antigen was diluted to standard 8 HA units in 50 µL. Serum samples were serially diluted 2-fold. Chicken red blood cells (RBCs, 0.5%) in PBS were used in the HI assay.

Virus quantification in oropharyngeal and cloacal swabs

Viral shedding was measured by calculating the 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. Next, 0.1 mL of each dilution was inoculated into the allantoic cavities of three 10-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 of incubation were discarded. Harvested allantoic fluid was tested for HA activity using 0.5% chicken red blood cells (RBCs) in PBS. The viral titers were calculated using the Reed & Muench method (Ramakrishnan and Muthuchelvan 2018).

Statistical analysis

Statistical analyses were performed using GraphPad Prism V. 6.0 software for Windows (GraphPad Software, San Diego, CA) and SPSS 16 for Windows (SPSS Inc., Chicago, IL). Student's *t*-test and Duncan's multiple range tests were used to compare the differences in means among groups. *P* ≤ 0.05 was considered significant.

Abbreviations

AIV: Avian influenza virus; LPAIV: Low pathogenic avian influenza virus; HPAIV: Highly 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 postvaccination; dpc: Days postchallenge; PBS: Phosphate-buffered saline; RBC: Red blood cells; BSL2: Biological Safety Level 2

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Authors' contributions

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

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Availability of data and materials

Data will be shared upon request by the readers.

Declarations

Ethics approval and consent to participate

All animal studies adhered to the 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 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.

Consent for publication

Not applicable.

Competing interests

The authors have no competing interests to declare. Author Zejun Li was not involved in the journal's review or decisions related to this manuscript.

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