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RESEARCH ARTICLE

In Ovo Vaccination against Avian Influenza Virus Subtype H9N2

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A B S T R A C T

Due to the major occurrence of infection by avian influenza virus (AIV) subtype H9N2 in broilers and broiler breeders at first 2-3 weeks of age in this country, where the virus is endemic since 1999, it has become necessary to devise new strategies for better protection of chicks at early age. *In ovo* vaccination is one of the approaches for providing neonatal resistance against various infectious diseases in chickens. The aim of this study was to develop *in ovo* vaccine against H9N2 serotype of AIV. For this purpose a vaccine strain of AIV H9N2 was used to develop both inactivated and live virus vaccines for experimentally inoculating18-day old embryos. In addition to this two other groups of chicken were separately vaccinated with both inactivated oil-emulsion and live virus vaccines of H9N2 for comparison. The hatched chicks were monitored for the development of HI antibody response against AIV H9N2. All these birds were later challenged with field isolate of AIV H9N2. The results indicated that *in ovo* live AIV H9N2 vaccine produced higher antibody response at younger age and showed better protection upon challenge.

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INTRODUCTION

Since the first introduction of low pathogenic avian influenza (LPAI) subtype H9N2 in Pakistan in 1999, it has become endemic in Pakistan especially in breeder-broilers and broilers (Naeem *et al.*, 1999). In the field conditions this subtype is incriminated in causing respiratory tract infection as early as 14 days of age and is involved in reproductive tract infection in laying bird during production, there by affecting egg quality, hatchability and production. Some studies have shown that several distinct lineages of AIV H9N2 exist in this region (Iqbal *et al.*, 2009). Such introduction of the new genotypes of H9N2 viruses not only lead to generation of more virulent strains of such influenza viruses, it may also result in reducing the efficacy of existing vaccines prepared from older AIV H9N2 strains.

Although immunization against AIV H9N2 has been established as one of the most promising control measures in poultry however a vast antigenic variation exist even within the same subtype (Iqbal *et al.*, 2009). Yet, as large-scale and long-term immunizations against AIV have been made in some countries, AIVs have also go through antigenic drift due to the presence of immune pressure (Hafez, 2011; Sun *et al*., 2012). This makes it very difficult to select a vaccine strain that can work as a potent vaccine in the face of widely circulating mutants of an AI virus.

A single administration of oil-based inactivated H9N2 LPAI vaccine is very immunogenic and highly protective in laboratory trials, use of that vaccine in the field induced poor antibody response due to environmental condition, farm stress and species of chickens (Lee *et al*., 2011). At present two types of AIV H9 vaccination schedules are used in broilers and laying hens in this country. This includes one shot of oil-based killed vaccine at the age of 4-6 day or one shot of alum adjuvanted vaccine 10-12 day of age. On the other hand in case of breeding stocks 2-3 shots of oil-based vaccines are used up to 20-week of age Despite the vaccination schedule, there are multiple reports of vaccine failure in the face of field challenge of H9N2 especially at early age of 14-18 days, indicating non-existence of sufficient antibody titers in the vaccinated chickens, or distinct mutation in the field strain.

In poultry, vaccines are usually administered as aerosols, oculo-nasal drops, through drinking water or by injection. With the advent of *in ovo* vaccination technique,

several advantages have been encountered over the above mentioned methods, like less neonatal resistance, administration of uniform dose of virus, and reduced handling charges by treating individual chick. *In ovo* vaccination of 18th day old embryo has been routinely experienced for the last two decades in commercial chickens in the US (Wakenell *et al*., 2002). Thus, mass delivery of new vaccines by this route can be easily adopted by the local industry. Especially in the current scenario of environmentally controlled housing system for broiler production in this country, it may be quite feasible to implement appropriate biosecurity measures to avoid the escape of live virus of a vaccine in the field.

Although live vaccination of LPAI type H5 and H7 is not recommended due to the possibility of its reversion to highly pathogenic avian influenza (HPAI), however no reversion of LPAI H9N2 to HPAI has so far been reported in literature. It has also been reported in the past that continuous circulation of LPAI H9N2 in the field and its controlled *in ovo* passaging up to 40 passages has not revealed any major mutation affecting its pathogenic potential (Choi *et al.*, 2008). The work reported here was, therefore, designed to assess the efficacy of a live AIV H9N2 vaccine in comparison with the inactivated oil-based vaccine, using *in ovo* route of vaccination.

MATERIALS AND METHODS

Source of virus: An isolate of AIV subtype H9N2, designated as A/Chicken/Pakistan/NARC-3272/08 (H9N2), was obtained from the repository of the National Reference Lab for Poultry Diseases (NRLPD), National Agricultural Research Centre (NARC), Islamabad, for use in this study. This isolate was propagated up to 13th passage level in 9-10 day old embryonated Specific Pathogen Free (SPF) chicken eggs as per earlier described procedure for the development of inactivated oil-based and live vaccines (Senne, 2008). Another field isolate recovered from an outbreak of H9N2 and designated as A/Chicken/Pakistan/NARC-7477/08(H9N2) was used as challenge virus in this study.

Embryonated chicken eggs and Day old chicken: For this study, SPF embryonated eggs were used for virus propagation for vaccine, however for the vaccine trial the embryonated chicken eggs (ECEs) and day old chickens were obtained from AIV- H9 vaccinated broiler breeder flock with no history of previous AIV infection. These chicks were raised under environmentally controlled and biosafe chicken isolators.

Production of Vaccines: For preparing inactivated vaccine, the vaccine virus was first propagated in 9-10 day-old SPF ECEs and the harvest was titrated in SPF ECEs to adjust the available virus concentration to 10^8EID_{50}/0.2 ml of vaccine dose (Reed and Muench, 1938). For killed vaccine, the harvested fluid was inactivated by treating with 0.1% formaldehyde at room temperature for 18 h at a shaker. The viral inactivation was later on confirmed by injecting the material in 10-day-old ECEs and by incubating at 37°C for 72 hr. The viral inactivation was verified by conducting Hem-agglutination test on the allantoic fluid recovered from the above referred eggs (Senne, 2008).The inactivated vaccine virus was subsequently emulsified with mineral oil adjuvant, following standard protocols (Stone *et al*., 1975). For preparing a live vaccine for AIV H9N2, the vaccine virus stock was adjusted to 10^3EID_{50}/0.01ml using normal saline solution for use as live vaccine.

Hemagglutination inhibition test: Hemagglutination Inhibition (HI) test was conducted according to the earlier described procedure (Pedersen, 2008). Briefly 25µl of phosphate buffer saline (PBS) pH 7.4 was dispensed into all the wells of U-bottom 96-well microtiter plate, followed by the addition of 25µl of test sera into firs well of row-A. After mixing, it is serially diluted by transferring 25ul to the next well in this row. Row-H of the microtiter plate will serve as positive control. Add 25µl of H9N2 antigen containing 4HA unit of the virus. Incubation of the plate for 30 minutes at room temperature (25°C) was followed by addition of 50µl of 0.5% chicken RBCs suspension into all the wells. Results were recorded after an incubation of 30 minutes at room temperature.

Experimental design for vaccination and challenge studies: Experimental Design for H9N2 vaccination: As shown in Table 1, chickens in group-A were tested upon using inactivated oil based vaccine in 18-day old embryos at a dose of 0.2ml/ embryo, via *in ovo* route. Whereas in the case of Group B chickens, only live AIV H9N2 vaccine was injected via *in ovo* route at a dose of 0.01ml/embryo. All the embryonated eggs in different groups were vaccinated by following a standard inoculation method (Ahmad and Sharma, 1992). In group C and D, the chickens were injected via subcutaneous and intranasal routes with inactivated oil-based vaccine and live vaccine, respectively. However, chickens in group E were kept as unvaccinated control.

In this study the parameters of performance of *in ovo* vaccination in terms of hatchability, chick's survival, production of antibodies and protection upon challenge were compared with conventional post-hatch vaccination data. For this purpose 2-3 ml blood samples were collected weekly from 5 chicks each time selected randomly from each vaccine group (Table 3) and sera were separated and centrifuged at 800xg for 10 minutes and subsequently stored at -80 C until used. HI test was performed on all serum samples using the antigen prepared from H9N2 virus used in the vaccine (Beard, 1980). The HI antibody titers were expressed as the reciprocal of the highest dilution of antisera showing hemagglutination. A geometric mean titer (GMT) for each group of sera was subsequently determined (Brugh, 1978).

Experimental challenge-protection study: At 49 days of age, all the birds in different groups were challenged using with a field isolate of AIV-H9N2 with a dose of 10^5 EID_{50}/0.2ml/bird. Following intranasal virus challenge the chickens were closely observed daily for clinical signs and symptoms of disease such as coughing, sneezing, swollen sinuses, anorexia and diarrhea. To isolate the challenged virus, oropharyngeal and cloacal swab samples from the challenged group were collected at 1, 5, and 10 days post infection (dpi) from all the chicks, suspending in 1 ml of virus transport media. The samples were subsequently inoculated into three 9-10 day-old SPF ECEs respectively, followed by assessment of the virus growth from the allantoic fluids as per procedure described by (OIE, 2012).

RESULTS

In ovo vaccination is an emerging trend in the poultry industry. The experimental data gathered in this study revealed that the vaccine virus used in this study had no negative effect on the hatchability of embryos upon *in-ovo* inoculation (Table 2). The percentages of hatched chicks and first week survival percentage from inoculated eggs of group A and B were close to the eggs hatched without any inoculation. Post-vaccination HI serology of various groups is depicted in Table 3. The data indicates sharp increase in antibody titers in all the groups from day 1 onward up to day 7 due to high absorption of maternal antibodies against AIV H9N2. Thereafter, as compared to all other groups, the group-B, which was given *in ovo* live vaccination, H9N2 antibody titer started stabilizing and eventually increased to significantly higher levels (Table 3). However the groups receiving inactivated *in ovo* vaccine started showing increase in antibody response against AIV H9 at 35th day post vaccination. On the other hand, HI antibody titers continued to increase in the live *in ovo* vaccine group (B). It was further recorded that the increase in antibody titer and its systemic decline was also not evident even in case of group D where live intraocular vaccination was used. It therefore, appears that *in-ovo* live vaccination is capable to significantly increase immune response against H9N2 in poultry, surpassing similar protection offered by other types of AI killed vaccines. These findings also indicate that the live vaccine virus was able to breach passive immunity barrier and seroconvert properly.

The data regarding post vaccination challenge in this study is depicted in Table 4. This reveals that better protection was obtained in the groups receiving *in ovo* live or inactivated vaccine as compared to other route and type of vaccines. Furthermore, only in group B, post-challenge shedding of AIV H9 was only detected on 5th day, whereas in rest of the groups this shedding was detectable both on 5th and 10th day post-challenge Table 5.

DISCUSSION

Avian influenza viruses (AIV) of the H9N2 subtype are prevalent worldwide being common in poultry population in Asia and the Middle East (Davidson *et al*., 2014). *In ovo* vaccination is a encouraging trend in the poultry industry because of its advantages like insignificant manpower involvement, induction of neonatal resistance and better protection. On the other hand, live vaccine failure is quite common in poultry due to non-maintenance of cold chains of vaccines, poor selection of vaccine strain, insufficient vaccine virus dose, presence of high levels of maternal antibodies in host, and faulty vaccination schedule. Chicken hatchability is an economically important parameter for the poultry industry. Previous studies have also shown that *in ovo* vaccines do not adversely affect hatchability or survival of hatched chicks (Riaz *et al.*, 2004).

Proper dose of vaccine virus to be administered *in ovo* is also critical for achieving satisfactory hatchability and immune response. However, administration of very low dose of vaccine may lead to insufficient antibody response (Ahmad and Sharma, 1992). For successful embryo vaccination, the selected vaccine dose should induce sufficient protective response in chickens having maternal antibodies against the vaccine virus. Such vaccine should also be safe for the embryos having minimum levels of maternal antibodies and should not hamper hatchability or survival of the chicks.

It therefore, appears that *in ovo* live vaccination is capable to significantly increase immune response against H9N2 in poultry, surpassing similar protection offered by other types of AI vaccines. Similar results were reported from some earlier studies on *in ovo* vaccination against MD and IBD (Zhang and Sharma, 2001).

Following *in ovo* administration, the vaccine virus replicates in embryonic tissues and this early virus replication leads to the development of protective

Table 1: Vaccine dose plan and route of vaccination in different groups on the basis of vaccine type

Vaccine type	Vaccine ID/Group	No. of ECE*/Chicks	Embryo/Chick age &Route of vaccination	Dose (ml)
Inactivated oil based	A	35 ECE	18 Day in-ovo	0.2
Live vaccine	B	23 ECE	18 Day in-ovo	0.01
Inactivated oil based	C	20 chicks	Subcutaneous	0.2
Live vaccine	D	20 chicks	intranasal	0.01
Control (non-vaccinated)	E	35 chicks	none	none

*ECE-Embryonated Chicken eggs.

Table 2: Effect of *in-ovo* vaccination of AIV subtype H9N2 on hatchability and first week survival of Hatched chicks

Vaccines	Egg type	No. of embryonated eggs used	No. of chicks hatched (%)*	Ist week survival rate (%)*
A	Commercial eggs	35	25/35 (71)	71
B	Commercial eggs	23	19/23 (82.6)	82.6
C**	Chicks	NA	NA	90
D**	Chicks	NA	NA	90
E	Commercial eggs	35	30/35 (85.5)	85.5

*Figures in parenthesis show % hatchability = (No. of chicks hatched/No. of eggs vaccinated) ×100); **Day old chicks used; % survival= (No. of chicks alive 7 days post-hatch /No. of chicks hatched) ×100; NA = Not Applicable.

Table 3: Vaccine efficacy against AIV subtype H9N2

	Serology(GMT)									
	Post-vaccination antibody titers							Post-challenge antibody titers		
Vaccine ID/Group	Day 1	Day 7	Day 14	Day 21	Day 28	Day 35	Day 42	Day 49	Day 56	Day 63
A	64	294.1	190	90.5	48.5	111.4	36.8	22.6	16	8
B	79	147	136	100	100	588.1	776	1024	724	32
C	-	-	126	119.2	112.6	117.7	24.3	38	14	4
D	-	-	116	127	119	112	24.3	16	14.9	4
E	90.5	207.9	32	22.6	21.1	13.9	14.3	6.1	32	64

*No. of samples tested on weekly basis: 5 samples/group randomly.

Table 4: Protection level of different groups after challenge

vaccine ID/ Group	No. of chicks challenged	No. of birds survived	% Protection
A	6	5	83
B	10	9	90
C	10	7	70
D	10	7	70
E	6	3	50

*% Protection= (Number of birds survived/Number of birds challenged) ×100.

Table 5: Post challenge AIV H9 Shedding in feces detected by in ovo inoculation

vaccine ID/Group	Dose/Bird 10^5 EID_{50}	Route of challenge	PC* Day 1	PC Day 5	PC Day 10
A	0.2ml/bird	intranasal	-	+	+
B	0.2ml/bird	intranasal	-	+	-
C	0.2ml/bird	intranasal	-	+	+
D	0.2ml/bird	intranasal	-	+	+
E	0.2ml/bird	intranasal	-	+	+

PC= Post-Challenge.

immunity at the time of hatching. Generally, higher the recoverable virus titer and longer the presence of virus in hatched birds, longer is the immunity (Zhang and Sharma, 2001). However, as the immune response elicited after live vaccine generally includes both humoral and cell mediated responses, therefore, simple determination of antibody titers against the live vaccine of H9N2 may not be reflecting true efficacy of this vaccine. However, it is quite evident from this study that use of live vaccine of AIV H9N2 may provide better, faster and prolonged protection against the field challenge of this virus.

Conclusion: *In-ovo* vaccination in poultry is an emerging trend in the poultry industry because of its several advantages of induction of neonatal resistance and better protection. This study was designed to compare the efficacy of live and inactivated oil based vaccines administered via *in-ovo* and sub-cutaneous routes. The data indicated high protective antibody titers against live vaccine of AIV H9N2 upon its in-ovo inoculation in broilers. This study suggests better efficacy of live AIV H9N2 vaccine administered through *in-ovo* delivery system of vaccination. The vaccine developed here from a well-adapted strain of AIV H9N2 has shown to be safe, immunogenic, and conferring a faster and prolonged immunity against the disease in commercial settings. This method of vaccination can also make it convenient to vaccinate large number of chicks at hatchery level, avoiding un-necessary handling of large commercial flocks at less conducive environment of a farm.

Author's contribution: SR, KN and NS conceived this study, SR conducted virus propagation, vaccine preparation, vaccination trial and serology. SR, KN, NS, MQ, MAA, AA, FR, SY helped in data interpretation. All the authors contributed in manuscript preparation and its final approval.

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