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Journal Article

POTENCY AND EFFICACY OF A LOW PATHOGENIC H5N2 INACTIVATED VACCINE AGAINST CHALLENGE WITH A MALAYSIAN H5N1 HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUS

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SUMMARY

The potency and efficacy of an avian influenza (AI) H5N2 inactivated vaccine that was developed at Veterinary Research Institute, Ipoh was tested. The percentage sequence identity of the HA gene of the H5N2 vaccine virus to the challenge virus [A/chicken/Malaysia/5858/04 (H5N1)] was 88.2% by nucleotide and 90% by amino acid sequences similarities, respectively. As for the HAI segment, the nucleotide sequence similarities were 88.3 % and by amino acid sequence 87.7%.For potency testing, the heterologous killed H5N2 AI vaccine, formulated as an oil emulsion was administered only once subcutaneously in twenty five two-week old commercial broiler chickens. The HI antibodies were not detectable at week 1 post vaccination. The HI GMT attained was 30, 63, 200, 54 and 32 by week 2, 3, 4, 5, and 6 post vaccinations. Efficacy study was conducted on ten SPF chickens at week 3 post vaccination. 60% of the birds (6/10) with HI titres ≥ 64 - 128 survived the challenged. H5N1 challenge virus was reisolated from all the birds with HI titre ≤ 32 that died, and each of the birds that survived with HI titres of 64 and 128, from the oropharynx and cloaca at day 3 post challenge. This vaccine protected 60% of chickens against mortality and did not prevent shedding after challenged with a HPAI H5N1 virus.

Keywords: Avian Infuenza, Virus, Vaccine

INTRODUCTION

Since the outbreaks of highly pathogenic avian influenza (HPAI) H5N1 in poultry in 2000 to 2004, various countries have adopted several strategies to control or eradicate the disease. Some have chosen stringent measures such as killing and destruction of infected poultry. However, as these methods proved to be expensive and biosecurity measures and culling cannot be implemented to successfully control or eradicate the disease for some countries, an alternative method, is therefore, vaccination. Vaccination is also one of the tools recommended by international health organisations in controlling AI (OIE). For this reasons only two types of vaccines have been currently approved, (i) heterologous low pathogenic inactivated vaccines and (ii) recombinant vaccines (Swayne *et al*., 2000). Since the emergence of H5N1 in Asia, several heterologous inactivated vaccines have been developed and tested against H5 and H7 influenza viruses in poultry and the use of heterologous inactivated H5N2 vaccines had been reported in chickens in Hong Kong (2002 - 2006), Pakistan (2006), India (2006), Russia (2005), Egypt (2006), in ducks, geese and chickens in China (2004) and Vietnam (2005) to name a few (Swayne *et al*., 2001; Swayne *et al*., 2006; Swayne 2009). Although these vaccines can protect poultry from clinical disease, sterile immunity is not achieved under field conditions, allowing for undetected virus spread and evolution under immune cover (Fuchs *et al*., 2009). However, controlling highly pathogenic H5N1 using inactivated highly pathogenic H5N1 vaccines are not permissible for fear that residual viruses that are not fully inactivated can cause outbreaks. Despite this, in 2003, Indonesia, however, started using an autologous inactivated H5N1 vaccine to control the rapid spread of H5N1 in its poultry population (Swayne, 2009). However, they showed that the inactivated homologous H5N1 vaccine being completely protective than the H5N2 virus vaccines against H5N1 challenged. In using inactivated heterologous vaccines, where the virus strain used to make the vaccine is of the same H subtype as the challenging field virus the clinical protection and the reduction or viral shedding are ensured by the homologous H group (Capua and Marangon, 2003). Similar HA subtype or high percentage homology (90 - 96%) between the vaccine strain and the circulating strain are critical factors for the efficacy of the vaccine. However, other factors such as antigen quantity and content and the adjuvant used for the efficacy of the inactivated vaccines are also important (Swayne *et al*., 1999; Wood *et al*., 1985). The ability of the heterologous vaccine to provide protection against mortality and morbidity, reduce cloacal and oropharyngeal shedding and ability to prevent viral spread to other vaccinated or susceptible birds have been considered as important factors for protective efficacy of the vaccine. The aim of the study is to determine the potency and efficacy of the inactivated H5N2 vaccine developed, and the ability of the vaccine to invoke sterile immunity as depicted by shedding of challenge virus, after challenged with a highly pathogenic Malaysian strain of H5N1 virus.

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MATERIALS AND METHODS

Viruses

The vaccine virus A/duck/Malaysia/8443/04 H5N2 was isolated from the cloacal swab of a duck in a routine surveillance study in the country. During isolation of the virus in 9 - 11 day-old SPF embryonated eggs, the HA activity was detected as early as the first passage. However, it took 4 passages before the virus kill the SPF embryonated eggs. The virus was non-pathogenic as determined by the intravenous pathogenicity index (IVPI) by the standard procedure (Council Directive 92/40/EEC (1992) Off. J. Eur. Communities L167, 1 - 16). The sequence of the HA cleavage site is TIGECPKYVKSDRLVLAKGLRNVPQ----RETRGLF.

The challenge virus strain used was A/chicken/Malaysia/5858/04 H5N1. This virus was isolated from chickens during an outbreak in Malaysia in 2004. The virus had an intravenous pathogenicity index (IVPI) of 3.0, where 4 weeks old chickens inoculated with this virus died within 24 hr (determined by AAHL, Geelong, Australia, the OIE Reference Centre for Avian Influenza). The presence of multiple basic amino-acids at the HA cleavage site sequence of

TIGECPKYVKSNRLVLATGLRNSPQRERRRKKRGLF

indicated the high pathogenicity of the virus. The lethal dose of the virus was determined to be $10^5 EID_{50}$/0.1ml where it causes 100% mortality of SPF chickens within 48 hr post-infection. All laboratory and animal experiments using the highly pathogenic H5N1 virus was performed in a BSL-3 facility of the Veterinary Research Institute, Ipoh, Malaysia.

Sequencing of the Haemagglutinin gene

PCR was carried out to amplify the full length HA gene of the H5N1 challenge virus A/chicken/Malaysia/5858/04 H5N1 and the A/duck/Malaysia/8443/04 H5N2, using HA specific primers as previously described (Hoffmann *et al*., 2001). The products were cloned into TOPO PCR vector and sequenced. Sequences were assembled and edited using Staden Package, Pairwise sequence alignments and the nucleotide and amino acid sequence were compared using the Bio-Edit 7 and Genetyx-Mac programmes.

Preparation of the H5N2 Vaccine

The master seed and working viruses of the duck isolate were prepared in SPF eggs. A preliminary batch of vaccine virus was produced by inoculating a batch of 500 SPF eggs with $10^{3.4}$ EID_{50}/0.1 ml (This dose was found to give the highest virus titre at day 3 post inoculation). Vaccine virus infected eggs were incubated for 3 days. The undiluted allantoic fluid containing virus was inactivated for 18 hr with B-propiolactone at 0.01 %v/v and adjuvanted with 10%Montanide™ gel. The pre-activation infectivity titre and the HA titre of the vaccine virus were $10^{7.3}$ EID_{50}/0.1 ml and 128 HAU respectively.

For determining the potency of the H5N2 inactivated vaccine, twenty five three-day old commercial broilers which were not vaccinated with any poultry vaccines were reared until they reached the age of two weeks-old. The birds were wing-banded and reared in a non-infectious animal housing unit.

Vaccination response-experiment

At two weeks old, the birds were immunized with the H5N2 vaccine. A dosage of 200µl was injected subcutaneously (SQ) per bird, and the serology of all the immunized birds were evaluated every week for a period of 6 weeks.

HI assay

The detection of antibodies after vaccination was studied by the HI assay performed according to the WHO manual on Animal Influenza diagnosis and Surveillance (WHO/CDS/CSR/NCS/2002.5). Serum samples were diluted 2 fold, with the initial serum dilution at 1:2. Titres > 3 $\log_2(8)$ are considered positive. The serological response was evaluated for all birds before and after vaccination. The HI test was performed in V-bottom 96 well microtiter plates with 8 HAU/50µl of homologous inactivatedH5N1 antigen per well.

Challenging vaccinated birds with H5N1 virus

In another experiment, ten two-week old SPF chickens (raised at SPF chicken facility of Veterinary Research Institute, Ipoh, Malaysia) were vaccinated with 200µl of the H5N2 vaccine via the SQ route. At 3 weeks post vaccination (based on 100% seroconversion from earlier potency study), the birds were challenged with 200µl containing $10^{5.3} EID_{50}$/bird of the H5N1 virus via the intranasal route. Challenging of the chickens with HPAI H5N1 virus, was conducted in a negative pressure isolator cabinet ventilated with HEPA-filtered air in a NATA-certified biosafety level-3 facility of Veterinary Research Institute, Ipoh. Water and feed were provided at libitum. Five SPF birds that had not been vaccinated with the H5N2 vaccine were also challenged with the same dose of virus. Clinical signs were monitored daily for one week post-challenged. Cloacal and oropharyngeal swabs of each of the chickens were sampled at 3 days post challenge for H5N1 virus re-isolation. Virus isolation was performed in 9 - 11 days old SPF embryonated eggs using standard procedures (OIE, 2012). The presence of H5N1 challenge virus was detected using the HA test and confirmed using specific H5N1 haemagglutination-inhibition (HI) serological test. Three passages were undertaken and HA test performed at each passage before the samples were considered negative.

RESULTS

HA gene sequence

Compared to the challenge virus, the percentage sequence identity of the HA gene of the vaccine H5N2 and challenge virus H5N1 was 88.2% by nucleotide sequence (Figure 1) and 90% by amino acid sequence. As for the comparison of the HAI segment, the nucleotide sequence similarities were 88.3 % and by amino acid sequence was 87.7% similarities.

Vaccination response

Table 1 and Figure 1, showed the HI GMT and the percentage of birds attaining positive HI titres at various weeks after a single vaccination dose with the H5N2 vaccine at two weeks old. By week 1 post vaccination (pv), HI antibodies were not detectable in any of the 25 vaccinated birds. By week 2 pv, 60% of the birds were positive (HI ≥ 8) for HI antibodies. By week 3pv, 100% of the birds seroconverted with positive HI titres; however, the titres were not high, where only seven birds had HI titres of 64 and 128. By week 4 pv, the percentage of birds with positive titre reduced to 96%, however, achieved the highest GMT of 200 where 18/25 birds (32%) attained high HI antibody titres of 64-512; and by week 6 pv, the antibodies waned off to a GMT of 32 with 72% of the birds having positive titre. However, the probable percentage of protection against mortality, based on a protective titre of ≥ 40 (Kumar *et al*., 2007), if birds were challenged with a pathogenic H5N1 strain would be 28%, 72%, 4% and 4% at week 3, 4, 5 and 6 post vaccination respectively (Figure 1).

Figure 1: Pairwise sequence alignment of the H5N2 and H5N1 HA gene showing homology in their sequence

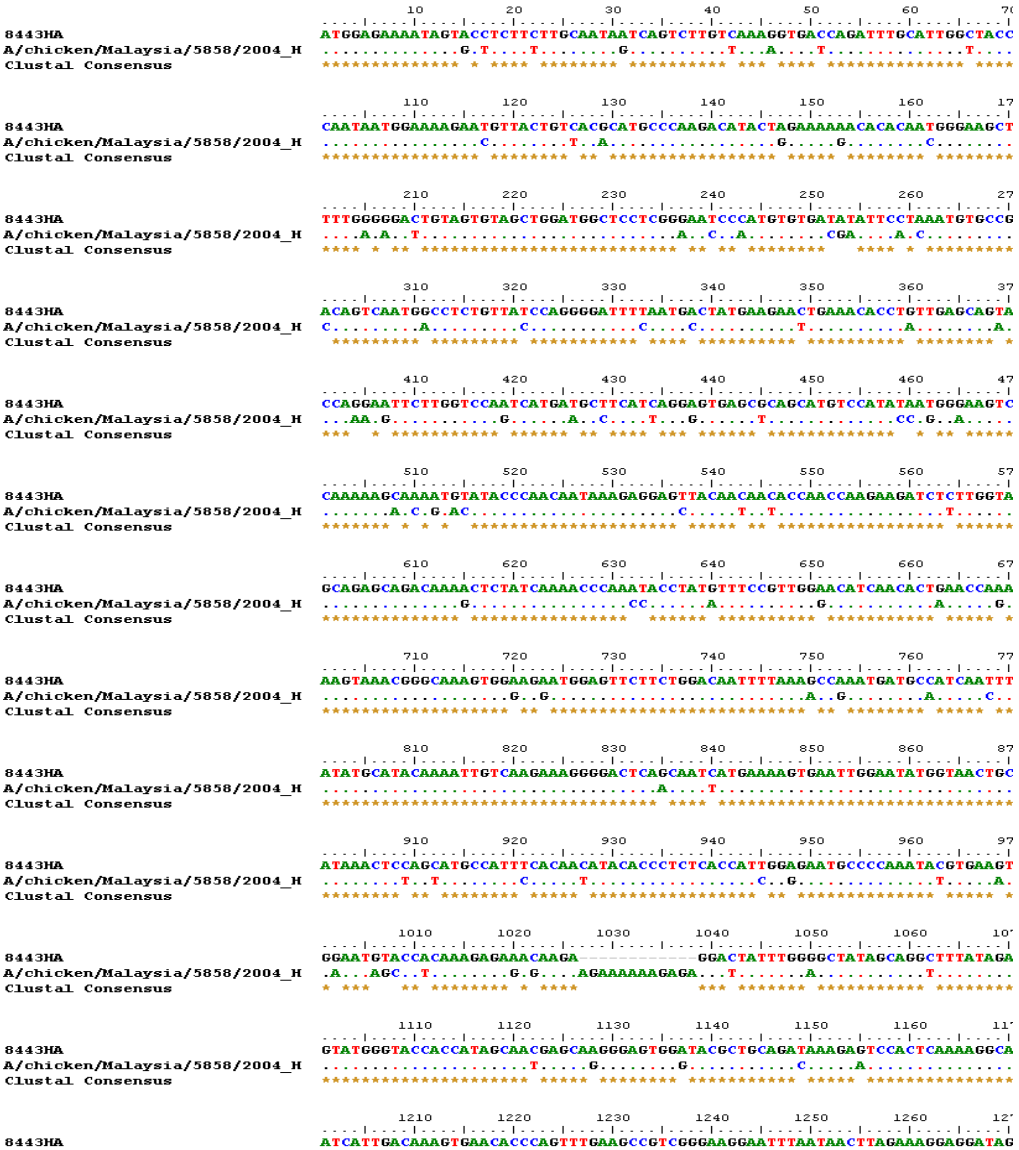

Table 1: Relationship of the potency, HI Geometric Mean Titre (GMT) and probable percentage protection afforded by the H5N2 vaccine on 25 commercial birds. Chicks were vaccinated at two weeks old and each bird was inoculated SQ with 200µl vaccine (pre-activation titre: $10^{7.3}EID_{50}$/0.1ml)

Week post vaccination	*No of birds*	*HI titre*	*GMT*	*a/b (Percent) positive HI titre :HI ≥ 8*	*Probable percentage of protection based on a protective titer HI value ≥ 40 (Kumar et al. 2007)*
0 (before vaccination)	25	<2	0	0/25 (0%)	0%
1	25	<2	0	0/25 (0%)	0%
2	5	<2	30	15/25 (60%)	0%
	5	4			
	5	8			
	7	16			
	3	32			
3	4	16	63	25/25 (100%)	7/25 (28%)
	14	32			
	5	64			
	2	128			
4	1	4	200	24/25 (96%)	18/25 (72%)
	2	8			
	2	16			
	2	32			
	10	64			
	6	128			
	1	256			
	1	512			
5	2	<2	54	22/25 (88%)	1/25 (4%)
	1	2			
	2	8			
	13	16			
	6	32			
	1	128			
6	4	<2	32	18/25 (72%)	1/25 (4%)
	1	2			
	2	4			
	12	8			
	5	16			
	1	64			

Challenged response and shedding

Only ten birds were used for the challenge and shedding studies as there was limited space in the BSL-3 cabinet for ease of handling the chickens. As was observed in the potency study, the rise of humoral HI antibodies were slow, i.e. it took three weeks post vaccination for all birds to seroconvert. Challenge was therefore done at week 3 pv, to ensure that all birds have antibody titres by then. The birds had pre challenged HI titres ranging from 8 – 128 i.e two birds with HI titre of 8, two birds with HI titre of 32; four birds with HI titre of 64 and two birds with HI titre of 128 (Figure 2). All four birds with HI titre ≤ 32 died during challenged. The birds died within 3 - 4 days post challenged. The six birds with HI titre ≥ 64 survived challenged with no clinical signs observed. Shedding was evaluated at only one time i.e. at 3 days post-challenged. Challenge H5N1 virus was excreted in the oropharynx and cloaca when examined at 3 days post challenged in 7/10 birds (70%), i.e from four birds that died at 3 - 4 days post challenged, in one bird with HI titre of 64 and one bird with HI titre of 128. Birds showed signs of depression, ruffled feathers and loss of appetite before death.

DISCUSSION

The Government of Malaysia does not adopt the policy of routine vaccination of poultry against avian influenza. However, in a worst case scenario, the government recognizes the potential of vaccination as a complementary measure in the control and eradication of HPAI, or at least for the vaccination of expensive or rare exotic birds. In view of this, a pilot batch of vaccine was prepared using a low pathogenic A/Duck/Malaysia/8443/04 (H5N2) virus. In our study, even at a high pre-activation titre of H5N2 virus of $10^{7.3}EID_{50}$/0.1 ml, and adjuvanted with 10% montanide gel (a potent adjuvant), the HI titres invoked with a single vaccination of this vaccine is moderately low

with the highest HI titre achieved was 512 in only one bird. It was only after three weeks post vaccination that 100% seroconversion was observed.

The HI antibody response could not be detected at 1 week post vaccination, however the GMT achieved its peak of 200 by week 4 pv but the antibodies waned off quickly by week 6 pv. This low-moderately low potency of the vaccine had also been shown by Kumar*et al*. (2007), in chickens vaccinated with a reverse genetic H5N3 isolate where the HA gene was derived from A/chicken/Vietnam H5N1. The chickens achieved suboptimal antibody response of HI < 40. He also showed that chickens with serologic responses of > 40 were protected against challenge with the H5N1 virus. He also showed that, at this protective titre, the virus could still be reisolated from one out of the 62 birds tested. In our potency study, using Kumar's value of HI > 40 as the protective titre, at week 3 and 4 post challenged, the probable protection afforded would only be 28% and 72% respectively. However, in our challenged study, using ten SPF chickens, 60% protection was afforded when chickens were challenged at week 3 post vaccination. We were also able to reisolate the challenge H5N1 virus in 7/10 birds. In conclusion, the H5N2 inactivated vaccine invoked only sub-optimal humoral HI antibody titres, not enough to protect at least 80% of the birds against challenge, although the HA protein share 90% amino acid homology with the challenge H5N1 virus. According to Swayne *et al*., 1999, the degree of protection of inactivated vaccines is not strictly correlated to the degree of homology between the HA gene or protein of the vaccine and challenge strains, therefore the vaccine can still be improved to achieve a higher degree of clinical protection and a better reduction of shedding i.e. by increasing the antigen mass of the vaccine. Due to space constrains of the BSL-3 facility, this is only a preliminary and small study, and therefore there were insufficient numbers of birds at all the various HI titres to make statistical inferences of protection associated with titres.

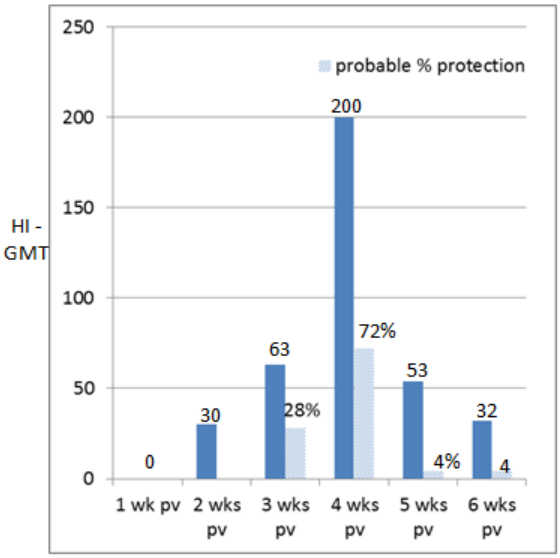

Figure1: Potency of the H5N2 vaccine. HI-GMT value and probable protection (HI > 40 based on Kumar *et al*., 2007)

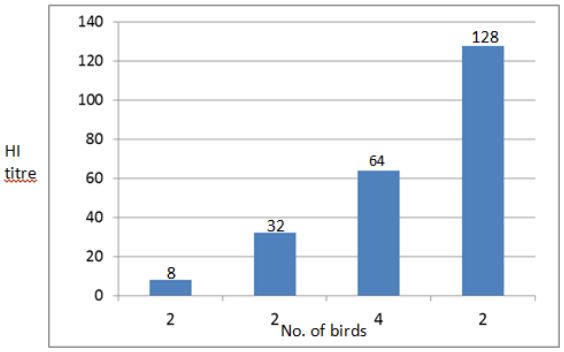

Figure 2: Challenged Study. 60% (6/10) of the birds was protected after challenged with the H5N1 virus. The protected birds had HI titres of 64 and 128

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