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## MODERATELY-HIGH HUMORAL ANTIBODY RESPONSES TO A H5N2 INACTIVATED VACCINE DID NOT SUPPRESS SHEDDING OF HIGHLY PATHOGENIC H5N1 AVIAN INFLUENZA VIRUS DURING CHALLENGE.

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### SUMMARY

Twelve SPF chickens with moderately high-titred HI antibodies of 7 log<sub>2</sub> and 8 log<sub>2</sub> achieved at week 4 post-vaccination after a single vaccination with an inactivated whole-H5N2 virus vaccine developed at Veterinary Research Institute, Ipoh were selected for efficacy and shedding studies. The H5N2 vaccine virus shares approximately 88.2% homology to the HA gene of the H5N1 challenge virus. The chickens with moderately high titred-HI humoral antibodies provided 100% protection against mortality and morbidity after challenged with a lethal highly pathogenic H5N1 Malaysian strain. The challenge H5N1 virus was reisolated from pooled cloacal swabs of chickens with HI titres of 7 log<sub>2</sub> and 8 log<sub>2</sub> at 3 days post challenged, however, was not reisolated from the pooled oropharyngeal swabs. The virus titre at reisolation was 10<sup>1</sup>EID<sub>50</sub>/0.1 ml in pooled cloacal samples from both the 7 log<sub>2</sub> and 8 log<sub>2</sub> HI titred-chickens. There was no challenge H5N1 virus reisolated from chickens with HI titre of 9 log<sub>2</sub>. This study demonstrated that chickens with moderately high HI humoral antibodies protect chickens against clinical disease and mortality did not fully prevent infection, however, was able to reduce virus shed via the cloaca and oropharynx.

Keywords: Avian Influenza, Virus, Vaccine

### INTRODUCTION

The H5N1 epizootic outbreak has resulted in the increase in the vaccination against H5N1 in poultry population of countries such as China, Italy, Mexico, Pakistan and Indonesia. For these countries, vaccination seemed to be one of the principal means of combating highly pathogenic (HP) avian influenza, as vaccination can control infection and reduce the incidence of clinical disease, thus reduce viral load in the environment (Swayne and Suarez, 2007; Capua *et al.*, 2007). As inactivated HP H5N1 vaccines are not feasible to be made into vaccines, due to reasons, such as incomplete inactivation which may result in disease and spread, and difficulty in differentiating from wild field and vaccine strains, inactivated vaccines based on reverse genetics and heterologous HA and NA antigens are therefore the best options. Inactivated vaccines, however, seemed to give variable results and boosters need to be given to achieve high immune responses. A study by Terrigino *et al.* (2006) showed that vaccination with an inactivated H5N9 subtype vaccine at 3 weeks and boosting at 7 weeks of age induced a very high immune response, GMT 10.3 log<sub>2</sub>. This immune response was protective against challenge with 10<sup>6</sup> EID<sub>50</sub>/0.1 ml of the highly pathogenic A/chicken/Yamaguchi/7/2004 H5N1 subtype and suppress shedding after challenge. Studies in ducks and geese also showed variable results. A study on an inactivated reverse genetic (RG) vaccine H5N1/PR8 for

ducks and geese, where the HA gene implicated in virulence is replaced with a non-pathogenic H5 gene, HI antibodies were only detectable at week 1 post vaccination (pv), and by 6 weeks pv, the antibody rose to a high HI titre of 1024 and waned off to a titre of 16 by 43 weeks (Tian *et al.*, 2005). A bivalent inactivated vaccine of H5N9 +H7N1 and a monovalent H5N3 which was given to 1 day old and 3 weeks old ducks, induced only low titres of 2 log<sub>2</sub> - 3 log<sub>2</sub> and 3 log<sub>2</sub> - 6 log<sub>2</sub> respectively, which was achieved at week 2 post vaccination. Despite the failure to stimulate significant HI titres, the bivalent vaccine did offer protection however, did not stop virus replication as seen in the seroconversion or the rise in antibody titres following challenge. The monovalent H5N3 vaccine, however, provided solid protection with no evidence of shedding of the challenge virus and no serological response to the H5N1 challenge virus (Middleton *et al.*, 2007). Lee *et al.* (2007), however, showed that doses or quantity of antigen of the same HA subtype and boosting are important for protective efficacy of the vaccine against H5N1 challenge. In their study, they showed that one dose of 128 HAU and 64 HAU homologous H5 vaccine induced 100% and 50% protection respectively. Virus shedding was prevented with the 128 HAU but not with the 64 HAU antigen quantities. They also showed that two doses at a 3-week interval with 64 HAU as well as an extra one dose of 1024 HAU of heterologous H5N3 vaccine provided 100% protection and prevent viral shedding completely. This is in agreement with studies by Swayne *et al.* (1999) who showed that there was a correlation between the antigen quantity or antigenic

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content and protective efficacy of the vaccine. In contrast, others reported that high virus titres can still be present in vaccinated chickens that are protected against clinical disease (Maas *et al.*, 2009) where in one study with H9N2 vaccine, after vaccination with as much as 128 or 1024 HAU low pathogenic (LP) H9N2 virus and despite the induction of high antibody titres, the LP H9N2 challenge virus could still be isolated from the vaccinated chickens (Choi *et al.*, 2008).

The molecular and antigenic similarities of 96.8 - 100% between the individual H5 avian influenza strains were sufficient to elicit solid cross protection against emerging HP H5N1 viruses, however, there was no positive correlation between sequence identity and the ability to reduce the quantity of challenge virus shed. For example, a study by Kumar *et al.* (2007) showed that chickens with high HI titres (> 640) elicited by a reverse genetics H5N3 avian influenza isolate and challenged with the original H5N3 virus i.e 100% similarity in the HA genes, did not result in sterile immunity as virus can be reisolated from at least 1/16 chickens with high antibody titres (HI:160 - 640).

The purpose of the study was, therefore, to determine the protection and effect on challenge virus shedding by chickens with moderately high titred-antibody as induced by a heterologous whole-H5N2 virus vaccine strain, with an HA gene homology of 88.2% with the challenge H5N1 virus. The practical implication of this trial is that, if protection is afforded and is effective in decreasing virus excretion at such moderately high titres, this vaccine need to be further improved eg. in its antigenic content during delivery, formulation with effective oil adjuvants and boosting effect, so as this vaccine can induced high titres in at least 80% of chickens in a single or multiple vaccination.

**MATERIALS AND METHODS**

*Viruses*

The Malaysian vaccine virus strain A/duck/Malaysia /8443/04 H5N2 isolated from ducks was developed as an inactivated whole-virus oil emulsion vaccine at Veterinary Research Institute, Ipoh. The pre-activation titre was 10<sup>7.3</sup>EID<sub>50</sub>/0.1ml. Cleavage site sequencing and the IVPI showed that the virus is of low pathogenicity. Table 1 showed the comparison between the H5N2 vaccine and H5N1 challenged strain. The challenge virus strain used was A/chicken/Malaysia/5858/04 H5N1.

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 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% (Sharifah *et al.*, 2012).

**Table 1: Characterization of the H5N2 vaccine virus strain compared to the challenged H5N1 virus**

<i>Comparison</i>	<i>Percentage HA gene</i>	<i>Sequence identity HAI segment</i>
Nucleotide sequence	88.2	88.3
Amino-acid sequence	90	87.7

*Vaccination of SPF birds and selection of chickens with high HI titre ≥ 128*

Thirty two-week old SPF chickens (raised in Veterinary Research Institute, Ipoh, Malaysia) were vaccinated with 200µl of the H5N2 vaccine via the SQ route. In our studies (Sharifah *et al.*, 2012) we found that 100% of birds had seroconverted by week 4 post vaccination and demonstrated the highest titres at this week of vaccination. At week 4 post vaccination, twelve chickens with high HI antibody titres of 7 log<sub>2</sub> - 9 log<sub>2</sub> (128 - 512) were selected, tagged and challenged with 200µl H5N1 virus by the intranasal route with 10<sup>6.0</sup> EID<sub>50</sub>/0.1ml of the virus. This dose was earlier shown to induce full mortality of unvaccinated controls with a MDT of 36 - 48 hr.

Challenged 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 VRI, Ipoh. Water and feed were provided at libitum. Three SPF birds of the same batch and age that had not been vaccinated with the H5N2 vaccine were used as the challenge control. Mean Death Time was determined for all birds that died.

*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<sub>2</sub> were 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 inactivated H5N1 antigen per well.

*Oropharyngeal and cloacal shedding 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 virus reisolation. The cloacal and oropharyngeal swabs of chickens with similar HI titres (i.e birds with HI titres of 7 log<sub>2</sub> (128), 8 log<sub>2</sub> (256) and 9 log<sub>2</sub> (512) were pooled and virus isolation performed in 9-11 day SPF embryonated eggs using standard procedures (Krauss *et al.*, 2004). Personnel of the Virology Lab of VRI who conducted the

re-isolation of the virus were blinded by the treatment group. The presence of H5N1 challenge virus in the allantoic fluid was detected using the HA test and confirmed using specific H5-haemagglutination-inhibition test. Three passages were undertaken and HA test performed at each passage before the samples were considered negative. Positive viral titres were expressed as EID<sub>50</sub>/0.1ml.

**RESULTS**

*Shedding of challenged virus*

Twelve chickens with high HI antibody titres of 7log<sub>2</sub>-9 log<sub>2</sub> (128-512) did not show any clinical signs and survived the challenged. Table 2 showed the HI titre at which the chickens were challenged, the morbidity, mortality and virus re-isolation data. H5N1 virus was however, isolated from the pooled cloacal swabs of the group with HI titre of 128 and 256, but not from the pooled oropharyngeal swabs. No virus was isolated from the pooled samples of the other two groups i.e. the group with HI titre: 256 and HI titre: 512. The H5N1 virus was only detectable at the third passage in SPF eggs and the titre was calculated to be 1 log 10 EID<sub>50</sub>/0.1ml. This shows that chicken/s in the HI titre: 128 and 256 group of chickens shed detectable amount of challenge virus by the cloacal route. This is a small study limited by space of the BSL-3 facility, to make statistical inferences of protection associated with these moderately high titres not possible.

**DISCUSSION**

From our previous study (Sharifah *et al.*, 2012), we showed that with a single vaccination of commercial birds with the inactivated whole H5N2 vaccine that was developed at VRI, Ipoh, the HI antibody titers did not rise to

high levels. The highest HI titre achieved was 9 log<sub>2</sub> (512) and only in 4% (1/25) of vaccinated chickens. Titres of 7-9 log<sub>2</sub> (128-512) was only achieved at week 4 post vaccination (Sharifah *et al.*, 2012). In this study, out of the 30 SPF chickens vaccinated only 40% (12/30) achieved a titre 7-9 log<sub>2</sub>. From the 30 vaccinated birds, only 5, 4 and 3 chickens attained an HI titre of 128, 256 and 512 respectively, at week 4 post vaccination. In this study, we inoculated 30 SPF birds with the aim of selecting moderately high HI titred chickens for efficacy and shedding evaluation. From the 30 vaccinated birds, only 5, 4 and 3 chickens attained an HI titre of 128, 256 and 512 respectively, at week 4 post vaccination. In this experiment we wanted to determine whether chickens with HI titres of 7-9 log<sub>2</sub> (maximum titres achieved using this vaccine in a single vaccination) can still protect birds against morbidity, mortality and shedding. There were no clinical signs observed in chickens after challenged. However, chickens seemed to excrete virus at 3 days post challenged via the cloacal route. The loads of virus isolated from the cloaca however, was very much reduced (10<sup>1</sup> EID<sub>50</sub>/0.1 ml) compared to the load of virus reisolated from the cloaca and also the oropharynx of the challenged control unvaccinated groups. This study confirms that heterologous vaccine and moderately high titred responses protected against clinical signs and mortality, and significantly decreased shedding after intranasal challenge, but they did not fully prevent infection or provide sterile immunity. This inactivated H5N2 vaccine sharing an 88.2 % nucleotide and 90% amino acid similarities with the HA gene and protein respectively of the challenge H5N1 virus, was able to protect chickens with moderately high HI titres of 7 - 9 log<sub>2</sub>. Although this vaccine protected chickens with moderately high titred-HI antibody against challenged, chickens with lower HI titres were not protected (Sharifah *et al.*, 2012).

**Table 2: Response of 12 SPF chickens (at 6 weeks old) with moderately high HI titres after a single vaccination with the H5N2 inactivated vaccine and challenged with pathogenic H5N1 virus**

<i>Challenged time</i>	<i>No of birds</i>	<i>HI titre</i>	<i>No. of morbidity</i>	<i>No of mortality</i>	<i>Isolation of H5N1 from pooled samples (titre of virus)</i>
Week 4 post-vaccination (6 weeks old)	5	128	0/12	0/12	H5N1 virus was reisolated at the third passage in SPF embryonated eggs (EE) only from the pooled cloacal swabs of chickens with HI titre of 128 and 256.  Virus load in the cloacal swabs from chickens of both HI titres was 10 <sup>1</sup> EID <sub>50</sub> /0.1 ml
	4	256			
	3	512			
Controls 6-wks old SPF chickens	3	< 2	3/3	3/3	H5N1 virus was reisolated from the pooled samples from cloacal swabs and oropharyngeal swabs of dead chickens.  H5N1 virus reisolated at 1st passage in SPF EE. Virus load was 10 <sup>3.6</sup> EID <sub>50</sub> /0.1ml

Boostering might result in chickens achieving much higher HI titres, however, this was not done for this vaccine.

All the studies showed that no matter how high the titre achieved by vaccination with a heterologous NA vaccine, high titres still cannot afford sterile immunity to all the birds. Some of the reasons for this could be due to such factors as host genetic and immune system, immunosuppression by other diseases the level of virulence of HPAI strains and partial cross protection, where for example, any one virus that escape the immunity, has the ability to multiply and replicate in the susceptible cells of the oropharynx and the intestines, i.e. areas not reachable by humoral HI antibodies.

In this study we were not able to isolate virus from the oropharynx, however, many workers including Swayne *et al.* (1999) and Kumar *et al.* (2007) were able to isolate the challenge virus from the oropharynx. The load of virus in the oropharynx was shown to be higher than in the cloaca. According to Swayne, a 100% homology between the haemagglutinin of vaccine and the challenge virus can protect birds against clinical disease but did not result in the prevention of infection by the challenge virus and shedding from the oropharynx. The differences in total amino acid sequence of the HA1 protein of vaccines, however, was also shown not to correlate with reductions in challenged virus titres shed from the oropharynx or cloaca.

Many workers have reported that vaccination cannot prevent infection whether the vaccine is heterologous or homologous to the virus challenged strain, therefore it is essential that as long as vaccination decreases virus excretion to levels that are insufficient for virus transmission within poultry flocks, than the vaccine should be acceptable. The variable results achieved by the various workers reflects the diverse variables used in each of the studies which include the use of different vaccines, and challenge viruses, different routes of administration of challenge viruses and the different doses and antigenic content of the vaccine at delivery.

Controlling of widespread transmission of the H5N1 virus is a major issue in countries where H5N1 is present and the use of inactivated vaccines is an effective control strategy (Swayne and Suarez, 2000). However, for eradication purposes, a mechanism for

the differentiation between infected and vaccinated chickens needs to be introduced (Capua, 2007). It is clear that, because of the human health implications of AI infections, control plans must aim at the elimination of the infection, based on any strategy that is chosen.

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