Journal of Animal and Veterinary Advances 10 (8): 999-1002, 2011
ISSN: 1680-5593
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Yeast-Derived Avian Influenza Virus Hemagglutinin Protein Induced Immune Response in SPF Chicken

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Abstract: Influenza pandemic virus appears to have originated from reassortment of inherited genes (HA, NA and PB1) from the avian influenza pool and genes from the human influenza virus. Specifically, regarding the low pathogenic Avian Influenza Virus (AIV), there is no commercial vaccine yet available. Hence, it is a priority to develop alow pathogenic avian influenza vaccine to help reduce morbidity and mortality outcomes during the next pandemic flu outbreak. In this study, the *HA* gene of an AIV A/Northern shoreler/AL/26/2006 isolate, AIV subtype H10N7 was expressed in the yeast *Saccharomyces pombe* and the recombinant protein was used to immunize Specific Pathogen Free (SPF) leghorn chickens held in Horsfall isolation units via the upper respiratory route at 1, 7 and 14 days of age. Serum and spleens of immunized chickens were analyzed and the results demonstrate that the yeast-derived recombinant HA protein is as effective as inactivated vaccine in inducing Hemagglutination Inhibition (HI) antibody, neutralization antibody and a Th1 like cytokine immune response. The results suggest that the recombinant HA protein could be a promising new subunit vaccine agent against low pathogenic AIV in chicken.

Key words: H10N7, influenza, vaccine, yeast, chicken, USA

INTRODUCTION

Since 2003, Highly Pathogenic Avian Influenza (HPAI) A/H5N1 viruses have been shown to be routinely transmitted from birds to humans often with fatal outcomes (WHO, 2008). Effective control of H5N1 epidemics requires both vaccination and antiviral-drug treatments.

However, influenza vaccines can lack efficiency due to antigenic variation. There is considerable interest in the development of new generation of influenza vaccines that are based on live attenuated viruses, virus like particles, virosomes, DNA vaccines and viral vectors (Kreijtz *et al*., 2009). As a result, many promising vaccine candidates are under investigation for HPAI. Low Pathogenic Avian Influenza virus (LPAI) can produce slight respiratory reactions in poultry but it can infect and recombine with IVs from other species resulting in Highly Pathogenic Influenza Virus (HPIV).

Hence, control measures must be developed to prevent the spread of AIVs (LP or HP) between species. Currently most conventional vaccines for the control and prevention of AIV outbreaks are focused on HP H5 and H7 AIVs. There exists a a need to elucidate the humoral and Cell-Mediated Immunity (CMI) responses against LP AIVs toward development of efficacious vaccines. The North American swine flu virus pandemic of 2009 contained *AIV* genes of unknown pathogenicity.

The *HA* gene of the H1N1 AIV from Alabama was 95% identical with the virus isolated from swine in Canada (Dormitorio *et al*., 2009). Based on the recent H1N1 pandemic influenza experience, there is need to develop a vaccine to prevent the spread of this and other AIVs between birds, swine and humans. Traditional vaccination methods for AIV requires costly and time-consuming injection of individual birds often multiple times to produce adequate protection. These vaccines are difficult to change quickly in response to new subtypes as manufacturing in embryos takes up to 6 months.

Yeasts are an ideal organism to express viral proteins because proteins can be glycosylated more accurately than bacteria, expression of recombinant proteins is rapid, inexpensive and yeasts are used as probiotics in animals and humans. Most species of yeast are safe for oral administration and allow expression to maintain immunity over time with no ill effect. In addition, yeast has natural

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adjuvant activity making expressed proteins more immunogenic when administered with yeast cell wall components. Researchers have produced recombinant HA protein in yeast and demonstrated its structural integrity (Wu *et al*., 2009b). As a continuation of the study, researchers investigate here the clinical immune response in Specific Pathogen Free (SPF) chicken caused by this yeast-derived HA protein.

MATERIALS AND METHODS

Virus and vaccine: Avian influenza virus subtype A/Northern shoreler/AL/26/2006 was isolated by researchers at Auburn University. This virus was isolated from blue-winged teal was passaged 4 times in SPF embryonated eggs and adapted to grow on Chicken Embryo Fibroblast cells (CEF).

The $TCID_{50}$ was determined as $10^{8.5}$ 50 μL^{-1}. Allantoic fluid was collected and used for viral RNA extraction with a Trizol RNA extraction kit (Invitrogen, Carlsbad, CA). The killed H10N7AIV vaccine and anti-H10N7 chicken serum was provided by Lohmann animal health international (Winslow, ME). Add brief information here on the recombinant HA protein expressed in *S. pombe*.

Vaccination of chickens: About 1 week old, SPF chickens were randomly separated into 5 groups (10 $group^{-1}$). Group 1 and 2 were controls and orally given 0.1 mL of saline or 0.1 mL of yeast culture; group 3 and 4 birds were given recombinant HA 25 or 50 µg, respectively at 1 week of age orally; group 5 birds were intramuscularly injected with 1 dose of inactivated H0N7 commercial vaccine Lohmann animal health international (Winslow, ME). Group 1-4 were treated at weekly intervals for 3 consecutive weeks. Serum samples were collected from the wing vein on days 0, 14, 28 and 49.

Serum samples were stored at -20°C until they were analyzed. Spleens from 3 chickens/group were collected at day 49 for cytokine analysis. The remaining 7 chickens were sacrificed and breast muscles were collected for immunohistological analysis.

Hemagglutination Inhibition (HI): For determination of Hemagglutination Inhibition (HI) titers, serum samples were heat inactivated at 56°C for 30 min prior to testing. Hemagglutination Units (HAU) of the AIV A/Northern shoreler/AL/26/2006 were determined before each assay using 2-fold dilutions. About 2-fold serially diluted sera in PBS and 4 HAU of AIV was used in a volume of 25 µL. The contents of each well were gently mixed with a micropipettor and the plates were incubated for 30 min at room temperature.

About 50 µL of a 0.5% chicken erythrocyte suspension was added to each well. The highest serum dilution capable of preventing hemagglutination was scored as the HAI titer. Data presented are the geometric means with standard deviation from three independent replicate experiments.

Virus eutralization ssay: Sera were analyzed for AIV-specific neutralization titers according to Robinson and Dowdle (1969). Briefly, serum or a heat inactivated monoclonal antibody at 56°C for 30 min was added to duplicate wells and serial dilutions were performed in the microtiter plates. All dilutions were made using MEM plus 2% FBS to a final volume of 75 µL per well. About 100 PFU of AIV in 25 µL were added to each well and the mixture was incubated at 4°C for 2 h followed by addition of 15,000 CEF cells in 100 µL of MEM plus 5% FBS to each well. Plates were incubated at 37°C in a CO_2 incubator for 3 days.

The plates were fixed by aspirating the contents of the wells washed 3 times with PBS at pH 7.2 with 0.5% Tween 20 followed by addition of 75 µL of an 80% (vol/vol) solution of acetone/PBS and incubation at 4°C for 15 min. After incubation, the contents were aspirated and the plates were air dried. ELISA was performed on the same plates with mouse anti-AIVF HA primary antibody (1:500 dilution) and Goat-anti-Mouse Ig-HRP (1:2000 dilution). OD values were read at 450 nm. The AIV-specific percentage neutralization titer was defined as follows: AIV (%) neutralization titer = (1 sample O.D. 450/RSV control O.D.450)×100%. The neutralization assay was performed in duplicate and data expressed as the means of two determinations.

Reverse ranscription and uantitative PCR (qRT-PCR) for cytokine analysis: Total RNA was extracted from chicken spleen using TRIzol (Invitrogen, Carlsbad, CA) according to manufacturer's instruction. About 5 µg of total RNA was treated with 1.0 unit of DNAse I and 1.0 µL of 10 x reaction buffer (Sigma, St. Louis, MO) incubated for 15 min at room temperature; 1.0 µL of stop solution was added to inactivate DNAse I and the mixture

Table 1: Sequence of the primers used in qRT-PCR

Name	Forward primer (5'-3')	Reverse primer (5'-3')	Accession No.
GAPDH[a]	GGTGGTGCTAAGCGTGTTAT	ACCTCTGTCATCTCTCCACA	K01458
IFN-γ[b]	AGCTGACGGTGGACCTATTATT	GGCTTTGCGCTGGATTC	Y07922
IL-2[c]	TCTGGGACCACTGTATGCTCT	ACACCAGTGGGAAACAGTATCA	AF000631
IL-4[d]	ACCCAGGGCATCCAGAAG	CAGTGCCGGCAAGAAGTT	AJ621735
IL-6[e]	CAAGGTGACGGAGGAGGAC	TGGCGAGGAGGGATTTCT	AJ309540
IL-10[f]	CGGGAGCTGAGGGTGAA	GTGAAGAAGCGGTGACAGC	AJ621614

[a] is the internal control(Wu *et al*., 2009a); [b] and [c] are the main Th1 cytokines (Romagnani, 1991; Sher and Coffman, 1992); [d, e] and [f] are the main Th2 cytokines (Sher and Coffman, 1992)

was hated at 70°C for 10 min. RNA was reverse transcribed using Superscript first-strand synthesis system (Invitrogen, Carlsbad, CA) according to manufacturer's recommendations. Oligonucleotide primers for chicken cytokines and GAPDH control were designed based on available sequences from public databases (Table 1). Amplification and detection were carried out using equivalent amounts of total RNA from chicken spleen using the QuantiTect SYBR Green PCR kit (Qiagen, Valencia, CA).

QPCR data analysis: The relative transcriptional levels of different genes were determined by subtracting the Cycle threshold (Ct) of the sample by that of the calibrator control GAPDH as per the formula: DCt = Ct (sample)-Ct (calibrator). The relative expression level of the specific gene in pcDNA6.2-HA vaccinated chicken compared to that in non-vaccinated chicken was calculated using the formula 2DDCt where DDCt = DCt (vaccinated)-DCt (non-vaccinated). Each analysis was performed in triplicate. Data were analyzed by Analysis of Variance (ANOVA) using SigmaStat statistical analysis software (Systat Software, San Jose, CA).

RESULTS AND DISCUSSION

Evaluation of yeast-derived HA caused humoral immune response in SPF chicken: After a single immunization with recombinant HA no antibody titers were detected in chicken with the Hemagglutination Inhibition (HI) assay. However, 40% of the animals developed Virus-Neutralizing (VN) antibodies against the homologous virus (H10N7). After the 3rd immunization, HI antibodies against H10N7 were detected in all birds (Table 2). The titer of VN antibody increased considerably and all animals had detectable titers against homologous strain (H10N7) while 60% of the chicken showed titer against the heterogonous strain (Avian H1N1).

Table 2: Evaluation of yeast-derived HA caused humoral immune response in SPF chicken

		Antibody response	
Vaccine groups	Virus	HI	VN
Gp1 (saline)	H10N7	0	0
Gp2	(yeast)	0	0
Gp3 (yeast-derived HA 25 μg)		100	100
Gp4 (yeast-derived HA 50 μg)		100	100
Inactivated vaccine		100	100
Gp1 (saline)	H1N1	0	0
Gp2 (yeast)		0	0
Gp3 (yeast-derived HA 25 μg)		60	60
Gp4 (yeast-derived HA 50 μg)		60	60
Inactivated vaccine		40	40

Proportion of animal (%) with detectable antibody titers (HI and VN) after 3rd immunization

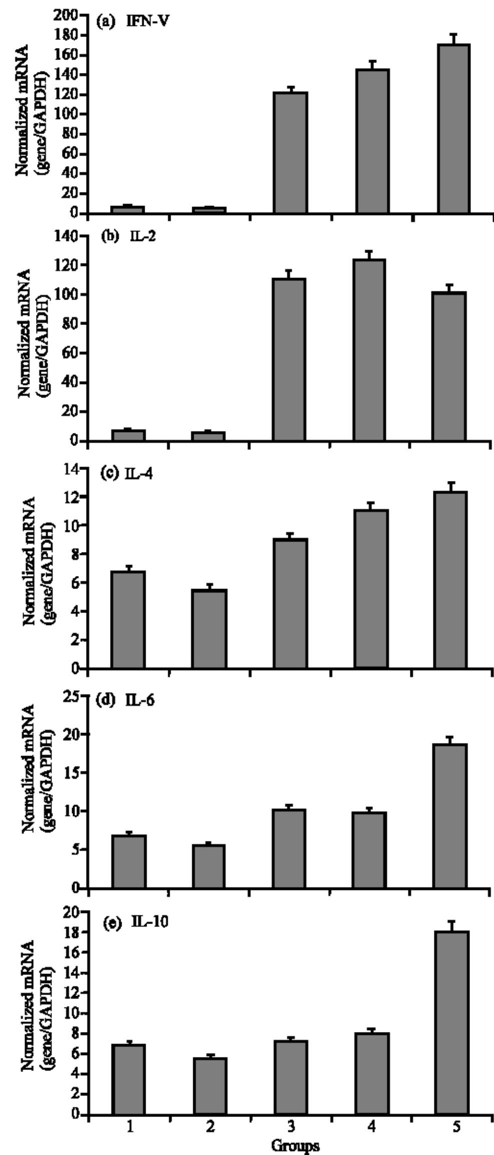

Fig. 1: Effect of yeast-derived HA on AIV-specific Th1/Th2 levels in immunized chickens. Chickens in group 1 and 2 were controls oral feed with 0.1 mL of saline and 0.1 mL of yeast *Saccharomyces pombe*, respectively; group 3 birds were orally given yeast-derived HA 25 μg at 1 week of age; group 4 birds were orally given yeast-derived HA 50 μg at 1 week of age. Groups 1-4 were all treated at weekly interval for 3 consecutive weeks; group 5 birds were IM injected with 1 dose of inactivated H10N7 vaccine. About 1 week after the 3rd immunization, the expression levels of mRNA in spleenocytes encoding chicken cytokines were quantified with Real-time RT-PCR

Cytokine analysis: The subsets of Th cells were distinguished by the cytokine production patterns. To distinguish between the activation of Th cells of the Th1 and Th2 subsets, the expression levels of mRNA encoding a panel of chicken cytokines were quantified in spleen lymphocyte following yeast-derived HA administration. Compared with negative controls, transcripts of the cytokines IFN-γ, IL-2 increased up to 50-fold following the 3rd oral feeding in chickens which received the high amount of HA (50 μg $dose^{-1}$). The concomitant increase of Th1 cytokine production was also significant (Fig. 1a-e).

The production of Th2 cytokines IL, IL-6 and IL-10 was not significantly different in any of the groups (1-5). The profile of cytokine response caused by yeast derived-HA was similar to the earlier study on a DNA vaccine (Wu *et al.*, 2009c). It is well established that Th1 cells produce IFN-γ and IL-2 which play a critical role in determining CMI responses. CMI is important for the clearance of intracellular pathogens.

The Th2 cells produce IL-4, IL-5, IL-10 and IL-13 which are associated with allergies and humoral responses (Romagnani, 1991; Sher and Coffman, 1992). The fact that yeast-derived HA protein could induce a Th1 preferred response suggests that it could be a promising vaccine candidate for LP AIV (Fig. 1).

CONCLUSION

The results presented in this study, characterize immune responses in SPF chicken caused by the yeast-derived HA protein including HI, VN and cytokine responses. The recombinant HA derived from *S. pombe* is active has a structural conformation of biological and immunological relevance and confirms the earlier study on the HA protein structure (Wu *et al.*, 2009b). Immunization with yeast-derived HA induced cross-reactive antibodies and cytokine response as effective as the commercial killed vaccine therefore, it is a promising candidate for the induction of protective immunity against low pathogenic avian influenza H10N7 viruses.

ACKNOWLEDGEMENT

This research was supported by Auburn University-Biogrant#: 101002121610-2052.

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