



## Lower Antibody Response in Chickens Homozygous for the Mx Resistant Allele to Avian Influenza\*

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**ABSTRACT :** The chicken Mx gene has been regarded as a candidate gene for resistance to avian influenza virus (AIV). In this study, three groups of chickens with homozygotes (AA, GG) and heterozygotes (AG) of the resistant (A) and susceptible alleles (G) to AIV of the Mx gene were constructed from a line of dwarf egg-type chickens. These chickens were not examined for their resistant activities to AIV because the differential resistance had only been detected *in vitro*. The birds of the three groups were vaccinated with inactivated H5N2 AIV vaccine and the level of hemagglutination inhibition (HI) antibody to AIV was detected. The association between disease resistant activity to AIV and antibody response to AIV vaccination in the three groups was analyzed. The chickens with homozygous resistant allele A showed the lowest antibody levels, whereas the heterozygous chickens (AG) presented the highest antibody level after the boosting vaccination, which indicates that the efficiency of artificial selection on the resistant allele of Mx gene will be compromised since the homozygotes of the allele presented the weakest antibody response to the corresponding vaccine. (**Key Words :** Antibody Response, Disease Resistance, Chick, Mx Gene, Avian Influenza)

### INTRODUCTION

There are two intriguing perspectives of anti-disease breeding including selection on disease resistant alleles of candidate genes and on antibody response to vaccines or antigens. Disease resistance has been extensively studied in a wide range of species including humans, mice, cattle, pigs and chickens (Arnheiter and Haller, 1983; McMichael and Bell, 1991; Bucin et al., 1992; Haller et al., 1998; Beuken et al., 1999; Rund et al., 1999; Flex et al., 2002; Dalgaard et al., 2003; O'Connell and McInerney, 2005; Quere et al., 2005), and many genes have been identified that contribute to disease resistance. The Mx protein is one of the best studied determinants of innate immunity and is considered to be involved in resistant activity to orthomyxovirus infection and has been found in many organisms including yeast (Rothman et al., 1990) and many vertebrates (Staheli

et al., 1989; Staeheli, 1990; Pavlovic and Staeheli, 1991; Bazzigher et al., 1993; Plant and Thune, 2004). In chickens, it is well documented that the Mx protein is associated with resistance to avian influenza virus (Flohr et al., 1999; Ko et al., 2002; Ko et al., 2004). The chicken Mx protein, which is a predominantly cytoplasmic form, consists of 705 amino acids (Bernasconi et al., 1995). Ko et al. (2002) have reported the polymorphisms and differential antiviral activity using 15 chicken breeds. They found a specific amino acid substitution at position 631 (Ser to Asn, S631N) to be associated with antiviral activity. After the 3T3 cell line had been transfected by different alleles of the Mx gene, allele A that has Asn at position 631 presented resistant activity to AIV, and allele G with Ser at the same position was susceptible to AIV. The frequency of the resistant allele was also found to be significantly different between native and highly selected commercial chicken lines, indicating the Mx gene could be positively selected in native chicken breeds due to its resistant activity (Li et al., 2006).

The humoral immune system that is mediated by serum antibodies secreted by B cells is one of the major immunological defenses. Genetic variation of the antibody response has been studied in various species (Biozzi et al., 1979; Siegel and Gross, 1980; Pevzner et al., 1981a; Pevzner et al., 1981b; van der Zijpp et al., 1983). The

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antibody response to the antigens including vaccines and pathogens is of importance in controlling infection. The selection for high or low antibody response to *Escherichia coli* at 10 d in broiler chickens was reported by Leitner et al. (1992), although the antibody response could be influenced by dietary in broiler (An et al., 2008).

The relationship between disease resistant activities and antibody responses against the corresponding pathogens or vaccines, however, is still poorly understood. In order to address this question, the antibody levels to inactivated H5N2 AIV vaccine were examined in three chicken groups with different Mx alleles which conferred differential antiviral activities to AIV.

## MATERIALS AND METHODS

### Chickens and genotyping

A segregation population at the S631N site of Mx protein was constructed in a line of dwarf egg-type chickens. Twenty-two males and 136 females that were genotyped as heterozygote (AG) at this site were used as parents to reproduce progeny with three different genotypes: AA, GG and AG. The genotypes of the chickens were screened using PCR single strand conformation polymorphism (PCR-SSCP) for the S631N position of the Mx gene. The method and the primers used here were the same as those described by Li et al. (2006). The mating was designed for minimizing the differences in genetic background other than the Mx locus among the chickens with the three genotypes.

The parental birds were vaccinated using the regular vaccination program of the layer, in which the birds were vaccinated with 0.3 ml of inactivated H5N2 AIV vaccine (Zhongmu, Beijing, China) at 18 weeks of age, and every 3 months thereafter. The fertilized eggs were collected from 60-62 wks. and 430 progeny were hatched and obtained at the same time. All of the progeny were raised in a conventional environment under the same conditions without any other vaccination than the H5N2 AIV vaccine. Simultaneously, five control chickens with AA genotypes were raised in the same environment without any vaccination.

### Samples collection, vaccination and antibody assay

Blood samples were collected via the wing vein for each progeny chick at 2 weeks of age, and genomic DNA was isolated from blood samples by the phenol-chloroform method (Li et al., 2006). For the progeny, all 430 chicks were screened by PCR-SSCP (Li et al., 2006) and divided into three groups based on the genotypes (AA, GG and AG) for the Mx protein. Fifty-eight individuals with AA, 56 with AG and 53 with GG genotypes were randomly selected from each genotype group to be examined for antibody

response. The ratio of the female and male was around 1:1 in all three groups. All selected experimental birds were vaccinated with 0.3 ml of inactivated H5N2 AIV vaccine (Zhongmu, Beijing, China) at 14 d according to the protocol provided by the manufacturer, and a boost vaccination was administered at 45 d. Blood samples (1 ml) were obtained from the wing vein from all chickens before the initial vaccination and at week 1, 2, 3, 4, 5, 7, 9 and 15 after the first vaccination. After the blood samples were centrifuged at 1,000×g for 10 min, the sera were collected and stored at -20°C. All serum samples were analyzed for the titer of hemagglutination inhibition (HI) antibody to AIV by HI test simultaneously. Since the H protein of AIV can hemagglutinate chicken red blood cell, the antibody to H protein of AIV can prevent the hemagglutination. Based on this rationale, we first mixed the 50 µl serum and serial half-diluted serum of each bird with 50 µl AIV virus solution for half an hour, and then put 50 µl of the mixed dilution into the same volume of chicken red blood dilution. The HI antibody titer was the highest times half-diluted serum solution which could prevent the hemagglutinating activity of AIV.

### Statistical analysis

The HI antibody levels to H5N2 AIV vaccine were compared among the chicken groups with the three genotypes of AA, AG and GG using the GLM procedure in SAS 8.2 (SAS\_Institute, 2001). We used the following model:

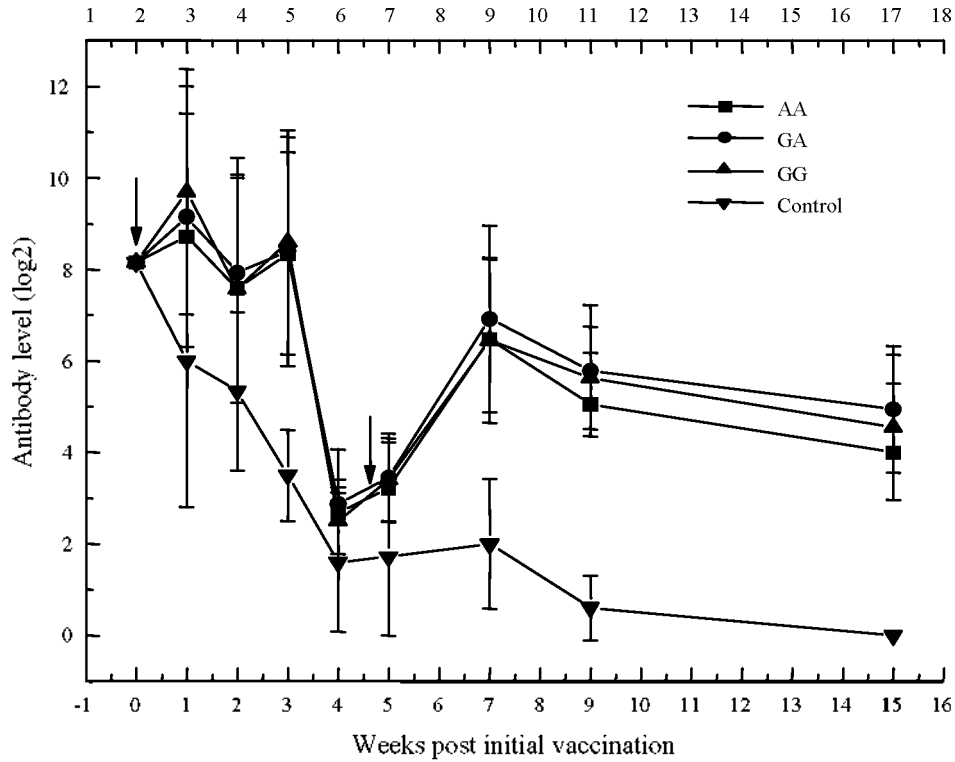
$$Y = \mu + s + g + e$$

where the Y is the HI antibody titer of each individual,  $\mu$  is the mean value of antibody titers of all chickens, s is the effect of sexes, i.e. female and male, g is the effect of genotype, and e is the random error.

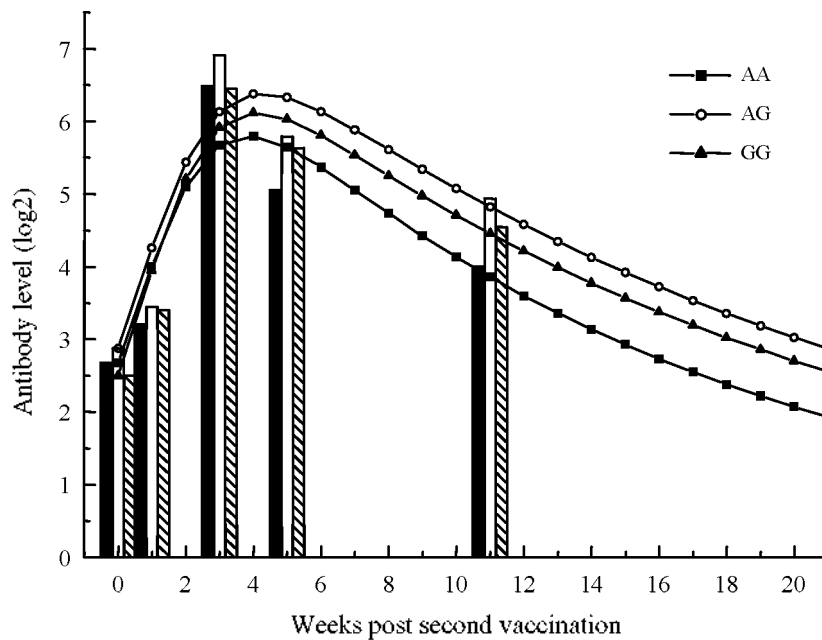
The curves of average antibody response were estimated using a nonlinear regression function which was initially mentioned by Weigend (Weigend et al., 1997). The NLIN procedure in SAS 8.2 and corresponding model used in Weigend's study (Weigend et al., 1997) were implemented for approximating the kinetics of AIV HI antibody in the three groups after second vaccination.

## RESULTS

The HI antibody levels detected in sera from the experimental chickens are shown in Figure 1. The post-vaccination antibody levels in all groups except for the control group changed with the same trend: during the first week after the initial vaccination, the antibodies quickly increased to high levels and then fluctuated in the next two



**Figure 1.** Mean values of HI antibody titers to inactivated H5N2 AIV vaccine of chickens with each genotype (AA, AG, and GG) of the Mx gene. Top X axis indicates the age of chicken and arrows show the times of initial and second vaccination. The points represent the levels of antibody that were detected at corresponding times using HI tests. Error bars are also shown for each genotype.



**Figure 2.** The simulated curves of the mean values of antibody titers to H5N2 AIV vaccine after the second vaccination in chickens of the three genotype (AA, AG, GG) groups. The bars represent the experimental data: Black bars indicate the antibody level of chickens with AA genotype; White bars indicate those with AG genotype and diagonal bars were those with GG.

weeks, to reach a minimum at the third and fourth week. However, no significant differences were found in the antibody levels in this period among the three groups.

After the boosting vaccination, the average antibody levels of chickens with heterozygous genotypes (AG) were always the highest while GG homozygotes presented the second highest and AA homozygotes showed the lowest antibody level. However, the differences among the antibody levels in the three groups were not significant until five weeks after the boosting vaccination.

In the control group, average maternal antibody levels were as high as 8 of HI antibody titer at hatch and thereafter decreased quickly. The high level of maternal antibody probably made the antibody levels to AIV untraceable after the initial vaccination. The maternal antibody decreased quickly after the initial vaccination and seemed to have no effect after the fifth week when the chickens were 7 wks old (Figure 1). The HI antibody titers to AIV after the boosting vaccination in the three groups were also estimated using Weigend's method (Weigend et al., 1997) and the results are shown in Figure 2. The maximum antibody level was found in the heterozygotes (AG) while the minimum was found in the AA genotypes after the boosting vaccination. The time for reaching the highest antibody levels was similar in all groups, namely about the fourth week after the second vaccination. The HI antibody titers in all groups and the comparisons among the antibody responses in the three groups are presented in Table 1. The standard deviations of antibody levels in all groups were high, which indicates that there could be many genes associated with antibody response to AIV besides the Mx gene.

## DISCUSSION

Mx protein has been found in a wide range of species and enables the resistant activity to orthomyxovirus (Haller et al., 1998; Lee and Vidal, 2002). In the present study, chickens with homozygotes (AA, GG) and heterozygotes (AG) of the Mx resistant (A) and susceptible alleles (G) to AIV were vaccinated with AIV vaccine and significant

antibody response differences were found among the three genotypic chicken groups five weeks after the boosting vaccination. We did not examine the disease resistant activities to AIV in the chickens because the chickens were randomly selected and also the anti-disease activities of the Mx gene had been only found *in vitro* (Ko et al., 2002, 2004). It was unexpected that chickens with homozygous resistant alleles (AA) showed the lowest antibody response to H5N2 AIV vaccine while those of the heterozygote (AG) presented the highest. Three possible explanations could account for this: i) The immune system is activated by different kinds of antigens. Vaccine commonly composed of proteins or peptides activates humoral immunity which mainly associates with antibody response, whereas virus mainly triggers cell-mediated immunity, in which Mx protein is an important GTPase induced by IFN which can stop the division of cells infected by viruses. So the antibody response to vaccine and disease resistant activity to virus are controlled by different genetic elements. ii) The chickens with the resistant allele are less reactive to AIV than those with the susceptible allele. So, the immune system with the resistant allele could react more slowly and weakly to vaccination. iii) In some other studies, the heterozygotes on anti-disease associated gene loci tend to show higher resistance to a certain disease than the homozygotes in chickens (Pevzner et al., 1981a; Pevzner et al., 1981b; Steadham et al., 1987; Wang et al., 2006). However, in those studies, chickens with homozygous MHC serotype that is susceptible to Marek's have almost no antibody response to another antigen, *S. pullorum* bacteria, even if the heterozygous chickens present distinctly higher antibody levels than homozygotes (Pevzner et al., 1981b). The advantages of a heterozygote on this locus of chicken Mx gene also partly contribute to the fact that the resistant allele A was not fixed in chickens (Flöhr et al., 1999; Ko et al., 2002).

The resistant allele of the Mx gene could be subject to positive selection (Jansa et al., 2003; Vallender and Lahn, 2004; O'Connell and McInerney, 2005; Hou et al., 2007) and much higher frequency of the allele was found in native

**Table 1.** Average HI antibody titers to H5N2 AIV vaccine of the three genotypes on the S631N site of the Mx gene. The values are all composed of average antibody levels and standard errors

Week of age	Weeks post first vaccination	Weeks post second vaccination	Average HI antibody titers to H5N2 AIV vaccine		
			AA	AG	GG
3	1	-	8.722±2.688	9.151±2.851	9.692±2.687
4	2	-	7.574±2.423	7.926±2.517	7.575±2.490
5	3	-	8.327±2.238	8.392±2.507	8.595±2.455
6	4	0	2.682±0.716	2.875±1.191	2.500±0.730
7	5	1	3.204±1.020	3.444±0.967	3.406±0.911
9	7	3	6.477±1.745	6.913±2.042	6.448±1.804
11	9	5	5.050±1.131 <sup>b</sup>	5.789±1.436 <sup>a</sup>	5.630±1.115 <sup>ab</sup>
17	15	11	4.000±1.497 <sup>b</sup>	4.936±1.389 <sup>a</sup>	4.545±1.583 <sup>ab</sup>

<sup>a, b</sup> Significant differences among average antibody levels in groups with different genotype ( $p < 0.05$ ).

chicken populations (Flohr et al., 1999). However, the homozygotes of the resistant allele A were found to present the lowest HI antibody titer to AIV after boosting vaccination, indicating the negative effects of the resistant allele A for the fitness of chicken to AIV, even if positive selection on this gene has been observed (Li et al., 2006; Hou et al., 2007; Berlin et al., 2008).

In this study, the homozygous disease resistant allele A of the Mx gene was found to present the lowest antibody response to corresponding vaccine. However, it is difficult to say whether this is similar to all other diseases because anti-disease traits are multi-gene controlled and too complicated to get universal rules. Further work needs to be done for different pathogens and other disease-related genes in different chicken strains or other species.

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