GENETICS

Associations of chicken *Mx1* polymorphism with antiviral responses in avian influenza virus infected embryos and broilers

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ABSTRACT Avian influenza virus (AIV) is a major respiratory disease of poultry that causes catastrophic losses to the poultry industry. The Mx protein has been shown to confer antiviral responses to influenza viruses in mice. One nonsynonymous substitution (S631N) in the chicken Mx protein is reported to be associated with resistance to AIV infection in vitro. The previous studies suggested controversy over whether this substitution in the Mx protein plays an important antiviral role in AIV infection in the chicken. It would be intriguing to investigate if the substitution is associated with resistance to AIV infection both in ovo and in vivo in chickens. In this study, the embryos and young chicks were generated from the cross of Mx1 heterozygous (S631N) parents with an expected segregating ratio of 1:2:1 in the progeny. A PCR length polymorphism was developed to genotype the Mx1 gene from 119 embryos and 48 chickens. The embryonated chicken eggs were inoculated with 10^6 50% embryo infectious dose (EID₅₀) H5N9 AIV on d 13. Hemagglutinating units in allantoic fluid were determined at 48 h postinoculation. For the in vivo study, twenty-four 1-wk-old broilers were inoculated with 10^6 EID_{50} H5N3, and virus titers in lungs were evaluated at d 4 postinoculation. This is the first report revealing no significant association between Mx1genotypes and low pathogenesis AIV infection both in ovo and in vivo in the chicken. Total RNA samples were isolated from chicken lung tissues in the in vivo study, and the Mx1 mRNA expression assay among 3 genotypes also suggested that only heterozygote birds had significantly greater expression with AIV infection than noninfected birds. A recombination breakpoint within Mx1 gene was also first identified, which has laid a solid foundation for further understanding biological function of the Mx1 gene in chickens. The current study provides valuable information on the effect of the Mx1gene on the genetic resistance to AIV in chickens, and Mx1 will not be applicable for enhancing genetic resistance to AIV infection in chickens.

Key words: chicken, Mx1 gene, low pathogenesis, avian influenza virus

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INTRODUCTION

Avian influenza virus (**AIV**) is a type A virus of the family *Orthomyxoviridae*. The Mx proteins are interferon-induced guanosine triphosphate enzymes and show antiviral activity for AIV in humans and mice (Haller and Kochs, 2002; Haller et al., 2007). The replication of influenza virus and other negative-strand RNA viruses were affected through the interruption of the viral transcription by Mx expression (Acheson, 2007). The Mx protein was reported to have intrinsic antiviral activity and be responsible for the influenza virus resistance in mammals (Arnheiter et al., 1990). In contrast to mammals, there is no consistent conclusion regarding antiviral activity of the chicken Mx1 gene in either in vivo or in vitro assays (Ewald et al., 2011). Chickens only have one Mx1 gene (Mx1), which was originally reported lacking antiviral activity (Bernasconi et al., 1995). Chicken Mx protein is encoded by Mx1 gene and is composed of 705 amino acids in which a tripartite guanosine triphosphate-binding motif and a leucine zipper motif is conserved among different species (Watanabe, 2007). There are several natural mutations in the chicken Mx1 gene. Interestingly, the Ser (S) to Asn (N) substitution at amino acid position 631 (S631N) caused by a SNP at nucleotide position 2,032 (G to A) of Mx1cDNA, had an antiviral activity in vitro on mouse 3T3

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cell lines (Ko et al., 2002, 2004). Mouse 3T3 cell lines expressing chicken Mx1 gene carrying N631 (AA genotype resistant allele) had significantly lower percentage of infected cells than those cell lines expressing S631 (GG genotype susceptible allele) Mx1 mRNA (Ko et al., 2004). A skewed allele frequency distribution in the S631N substitution was observed in different chicken populations, in which the viral resistant amino acid As had a much higher frequency in Chinese native chicken breeds than in highly selected commercial lines (Li et al., 2006). However, conflicting results have been reported in terms of the antiviral capability of the N631 polymorphism. The N631 allele of chicken Mx gene was not able to inhibit AIV replication in chicken primary embryo fibroblast cell lines (Benfield et al., 2008). Schusser et al. (2011) demonstrated that the expression of N631 and S631 Mx isoforms in chicken embryo fibroblasts were not associated with interferon-mediated resistance to influenza virus infection (Schusser et al., 2011). Most studies in Mx1 antiviral function have been focused on in vitro. To fully understand the antiviral activity of Mx protein, we sought to evaluate whether the Ser to Asn substitution in the Mx1 gene is associated with antiviral activity in vivo and in ovo in a unique population derived from an intercrossed Mx1 S631N heterozygous birds.

MATERIALS AND METHODS

Experimental Inoculation of Embryos and Chickens

Embryos and chickens were generated from the cross of Mx1 heterozygous (S631N) broiler parents. The Mx1cross was made in a large pen containing 70 females and 10 males (a ratio of 7 females to each male in colony mating). The breeders were from a commercial pedigree broiler line and identified as heterozygotes; therefore, the expected segregating ratio was 1:2:1 in the progeny. A total of one hundred nineteen 13-d-old embryonated chicken eggs were inoculated with 10⁶ 50% embryo infectious dose (**EID**₅₀) H5N9 AIV. Hemagglutination assay was used to evaluate virus replication in chicken embryos. Hemagglutinating units (**HAU**) in allantoic fluid were determined at 48 h postinoculation for all infected embryos. The DNA isolated from leg muscles was used for Mx1 genotyping.

For the in vivo challenge study, birds were housed in negative pressure Horsfall-Bauer temperature control isolation units and provided with water and commercial feed ad libitum. Chicken combs on d 1 were collected to isolate DNA samples for Mx1 genotyping. Sixteen chickens from each genotype were used. At 1 wk of age, 8 chickens from each genotype were inoculated with 0.2 mL of CK/TX/02/H5N3 virus containing $10^6 \text{ EID}_{50}/\text{mL}$, whereas the remaining 8 chickens were inoculated with PBS (mock inoculation) by the intrachoanal cleft route. All birds were killed at 4 d postinfection (**dpi**), and chicken lung samples were collected for RNA isolation. Virus replication at 4 dpi in the lung was determined by real-time reverse-transcription (**RT**) PCR for influenza matrix gene (M protein) using the AgPath-ID AIV-M kit (Ambion, Austin, TX) following the manufacturer's instructions. Control RNA was extracted from serially diluted H5N3 virus ($10^{1.5}$ to $10^{5.5} \log_{10} \text{EID}_{50}/\text{mL}$). A standard curve was generated with control viral RNA, and the amount of viral RNA in the samples was converted into $\log_{10} \text{EID}_{50}/\text{mL}$ by interpolation as described previously (Lee and Suarez, 2004). The animal experiment was performed according to the guidelines approved by the Institutional Animal Care and Use Committee, Texas A&M University.

Genotyping and Sequencing

Genomic DNA was extracted from the embryonic leg tissues or chick combs using Wizard Genomic DNA purification kit (Promega, Madison, WI) following the manufacturer's protocols. Genotyping of S631N was carried out by PCR length polymorphism (**PCR-LP**). The PCR primers (+MX1SER: 5' GCTCTCCTTG-TAGGGAGCCAG 3'; +MX1ASN: 5' TAATAATA-ATAACCTCTCCTTGTAGGGAGCGAA 3'and -MX1SERASN: 5' GTGACTAATTCTGCTGGTCAG-TAAC 3') were designed to amplify a fragment in the coding region of chicken Mx1 mRNA sequence (accession no. Z23168) including the substitution S631N. The PCR conditions were 94°C 2 min, followed by 35 cycles at 94°C for 30 s, 55°C for 1 min, 72°C for 1 min, and a final extension at 72°C for 7 min. The PCR products were then loaded on Synergel (0.7% agarose and 1.66%Synergel) and run in a Tris-acetate-EDTA buffer at 110 V for 5 h. Different genotypes were determined by the size of PCR products due to the different sizes of forward primers for alleles G and A.

To identify other Mx1 mutations in the coding region besides the S631N substitution (the mutation G2032A on the nucleotide sequences), total RNA isolated from chicken lung samples was reverse transcribed into cDNA by random hexamers using the Thermo-Script RT-PCR system (Invitrogen, Carlsbad, CA) in a reaction volume of 20 μ L. Using these cDNA as template, the complete coding region of the chicken Mx1gene (accession no. Z23168) was amplified by 3 pairs of primers (P1F1:5' GCTCGGTGCAGTACCTGCGG 3', P1R1: 5' TTCCCCACGGCCTCTCTGGC 3'; P2F2: 5' GCCAGAGAGGCCGTGGGGAA 3', P2R2: 5' CCC-GTCCGCGGTACTGGTCT 3'; P3F3: 5' CCAGTAC-CGCGGACGGGAGT 3', P3R3: 5' GGTTGCTGCTA-ATGGAGGATTTTGC 3'). The PCR conditions were $94^{\circ}C$ 7 min, followed by 35 cycles at $94^{\circ}C$ for 30 s, $55^{\circ}C$ for 1 min, 72°C for 1 min, and a final extension at 72°C for 10 min. The PCR products were purified by PCR purification kit (Qiagen, Valencia, CA). Purified PCR products were sent to the Gene Technologies Laboratory at Texas A&M University to do sequencing with Perkin Elmer ABI Big Dye reaction by the ABI 3100 Automated Sequencer (Life Technologies, Carlsbad, CA).



Figure 1. Polymerase chain reaction length polymorphism (PCR-LP) genotyping of the chicken Mx1 gene. Examples of PCR-LP products by Synergel gel (Life Technologies, Carlsbad, CA) separation. GG: homozygous G allele; AA: homozygous A allele; GA: heterozygous. N = Asn; S = Ser.

Gene Expression

Primers (forward: 5'GCACACACCCAACT-GTCAGCGA 3';reverse: CCCATGTCC-5'GAAACTCTCTGCGG 3') were designed to examine chicken Mx1 gene expression by real-time PCR with an amplicon of 157 bp across both exons 10 and 11. The reverse-transcripted cDNA was used as a template. The PCR reactions were performed in a $10-\mu L$ volume containing a $1 \times \text{SYBR}$ Green Master Mix on an ABI Prism 7900HT sequence detection system (Applied Biosystems, Foster City, CA). The amplification conditions were 50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 59°C for 1 min, and a final soak at 4°C. Chicken β -actin gene (forward: 5' ACG TCT CAC TGG ATT TCG AGC AGG 3'; reverse: 5' TGC ATC CTG TCA GCA ATG CCA G 3') was amplified by the same amplification condition and was used for normalization. The expression levels of chicken Mx1 were measured in terms of threshold cycle value (**CT**) and normalized to β -actin using $2^{-\Delta CT}$ (Schmittgen and Livak, 2008).

Statistical Analysis

Data were subjected to one way ANOVA of JMP 8.0 (SAS Institute Inc., Cary, NC). A significance threshold of P < 0.05 was considered statistically significant.

RESULTS AND DISCUSSION

A PCR-LP method followed by Synergel gel separation was developed to identify 3 different Mx1 genotypes. Two forward primers (+MX1SER and +MX-1ASN) with the same reverse primer (-MX1SERASN) generated different sizes of PCR products based on which allele (A or G) was present in the sequence. The PCR products for genotypes AA (allele A resulting in amino acid N) were 211 bp long, and 199 bp for genotype GG (allele G resulting in amino acid S), and 2 bands for the heterozygous GA genotype (Figure 1). With a total of 119 chicken embryos, 25 NN, 63 NS, and 31 SS genotypes were identified, which did not significantly deviate from the expected ratio of 1:2:1.

Chickens with the AA genotype were considered resistant, whereas birds with the GG genotype were considered as susceptible (Ko et al., 2002, 2004). Virus titers (HAU) for chicken embryos are shown in Figure 2. At 48 h postinfection, GG birds had a higher virus titer than AA genotype birds, although the difference was not significant (P > 0.05). For young chicks, there was a similar tendency with GG genotype birds having the highest virus titer $(\log_{10} \text{ of the tissue culture})$ infectious dose 50) followed by GA and AA genotypes (Figure 3). Our results show that chickens with AA genotype had a tendency for lower virus titers than GG birds both in ovo and in vivo, although the differences were not significant (P > 0.05). The sample size can affect significance level in a given experiment. We used 119 chicken embryos and 48 chicks in the current study. The relatively small population sizes, especially for the chick animal trial, plus standard variation among individual chickens need be taken into account for the reason why the differences were not significant.



Figure 2. Virus titers of different Mx1 genotypes in chicken embryos. HAU = hemagglutinating units.



Figure 3. Virus titers of different Mx1 genotypes in chickens.

TCID50 = the tissue culture infectious dose 50.

Table 1. Genotypes of identified single nucleotide polymorphisms in the coding region of chicken Mx1 cDNA sequences

S631N (G2032A)	A61G	G121C	C124T	A155T	A280G	T792C	A813G	G922A	A1015G	A1545G	A1747G	G2032A
GG	AA	GG	CC	AA	AA	CC	GG	AA	GG	GG	GG	GG
GG	AA	GG	$\mathbf{C}\mathbf{C}$	AA	AA	$\mathbf{C}\mathbf{C}$	GG	AA	GG	GG	GG	GG
GG	AA	GG	CC	AA	AA	$\mathbf{C}\mathbf{C}$	GG	AA	GG	GG	GG	GG
GG	AA	GG	CC	AA	AA	CC	GG	AA	GG	GG	GG	GG
GG	AA	GG	CC	AA	AA	CC	GG	AA	GG	GG	GG	GG
GG	AA	GG	CC	AA	AA	CC	GG	AA	GG	GG	GG	GG
GA	AA	GG	CC	AA	AA	TC	AG	GA	AG	GA	AG	GA
GA	AA	GG	CC	AA	AA	TC	AG	GA	AG	GA	AG	GA
GA	AA	GG	CC	AA	AA	TC	AG	GA	AG	GA	AG	GA
GA	AA	GG	CC	AA	AA	TC	AG	GA	AG	GA	AG	GA
GA	AA	GG	CC	AA	AA	TC	AG	GA	AG	GA	AG	GA
GA	AA	GG	CC	AA	AA	TC	AG	GA	AG	GA	AG	GA
AA	GG	CC	TT	TT	GG	TT	AA	GG	AA	AA	AA	AA
AA	GG	CC	TT	TT	GG	TT	AA	GG	AA	AA	AA	AA
AA	GG	CC	TT	TT	GG	TT	AA	GG	AA	AA	AA	AA
AA	GG	CC	TT	TT	GG	TT	AA	GG	AA	AA	AA	AA
AA	GG	$\mathbf{C}\mathbf{C}$	TT	TT	GG	TT	AA	GG	AA	AA	AA	AA
AA	GG	CC	TT	TT	GG	TT	AA	GG	AA	AA	AA	AA

Genetic resistance is a quantitative trait with multiple genes contributing toward resistance (Ewald et al., 2011). Genetic resistance to avian viruses has been studied for decades and differences in genetic susceptibility or resistance to major viral pathogens such as avian leukosis and Marek's disease viruses have been known to exist in poultry (Bumstead, 1998). The antiviral activity of the chicken Mx protein has been reported to be associated with amino acid variation at position 631 (Ko et al., 2004). Skewed allele frequencies of the Mx1 at position 631 have been found in different chicken genetic lines with different characteristics of resistance to pathogen infection, which suggested this Mx1 variation might be associated with AIV infection (Ko et al., 2004; Li et al., 2006; Yin et al., 2010a). Several experiments have been attempted to examine the associations between AIV infection and genotypes (S631N) of the chicken Mx1 gene. A recent study has indicated Mx1 N631 was associated with morbidity, early mortality, and viral shedding in a highly pathogenic AIV-infected chickens; however, such an association had not been confirmed either in the experiment with highly pathogenic H7N1 virus (Sironi et al., 2011) or in vitro using low pathogenic AIV (Ewald et al., 2011). No previous study has been conducted in ovo and in vivo with low pathogenic AIV infection in the chicken. In the current study, no such significant associations both in ovo and in vivo were identified. The Mx1 gene is very polymorphic in the chicken (Ko et al., 2002). We speculated that other coding region mutations besides amino acid 631 might contribute to AIV resistance in the chicken. Three pairs of primers were designed to amplify the entire coding region of the chicken Mx1gene, and PCR products were sequenced to screen for additional mutations. Besides the substitution at amino acid 631 (nucleotide G2032A), 11 additional point mutations were identified within the chicken Mx1 coding region, and genotypes of these mutations are presented in Table 1. Of particular note, genotypes on the mutations after T792C (the gray area in Table 1) were cosegregated with each other, whereas genotypes on the mutation before T792C (the white area in Table 1) of all heterozygote individuals in S631N shared the same homozygote genotype with GG (S631N) individuals, which suggested there was a recombination breakpoint between positions 280 and 792. To our knowledge, this is the first report on the recombination identified within the Mx1 gene in animals.

The Mx1 mRNA expression increased in chicken embryo fibroblasts with the treatment of poly I: C (Yin et al., 2010b). In the current study, we examined chicken Mx1 mRNA gene expression levels in the 3 Mx1 genotypes (S631N) by real-time RT-PCR. The Mx1 gene expression levels in AIV infected and noninfected chickens are shown in Figure 4. The Mx1 mRNA expression was greater in AA genotype birds than GG birds in AIV-infected or noninfected chickens, although the difference was not statistically significant due to great variation within the group (P > 0.05). This tendency was consistent with a previous study in which chickens of the AA genotype had greater Mx1 expression levels than the GG genotype in both Beijing-You and White Leghorn lines (Yin et al., 2010b). With AIV infection, Mx1 mRNA expression in chickens of only the GA genotype, not both homozygote (AA and GG), was significantly upregulated compared with the noninfected ones (P < 0.05). The exact mechanisms why mRNA level in both homozygote birds with AIV infection were not significantly regulated remains unclear. Based on Mx1coding sequencing information obtained in this study, for GA (S631N) birds, we assume all sequences including promoter region before position 280 were the same as GG genotype birds. We speculate sequence variation after position 280 might be responsible for gene expression regulation difference in GA genotype birds. Therefore, further investigation on other regulatory elements including 3' untranslated regions of chicken Mx1 gene is warranted.



Figure 4. The Mx1 mRNA expressions of infected and noninfected birds with different genotypes. Asterisks (*) indicate significant difference between infected and noninfected group within each genotype (P < 0.05).

There has always been a debate on whether the S631N substitution in the Mx protein plays an important antiviral role in AIV infection in the chicken (Acheson, 2007; Benfield et al., 2008; Daviet et al., 2009). Different anti-viral responses to high pathogenic AIV infections among different genetic lines were found, and no significant association of Mx1 polymorphism with the resistance to AIV infection was observed using a whole genome association assay (chicken 60K Array) in a recent study (Sironi et al., 2011). Another report showed that the antiviral effect of type I interferon in chicken embryo fibroblast cells was not dependent on the Mx protein, suggesting that chicken Mx1 might not be critical in the inhibition of AIV replication in chickens (Schusser et al., 2011).

In addition, genetic background might play a significant role in the anti-AIV response in the chicken. In 2007, Mx1 polymorphisms of 294 samples from 37 strains of 17 chicken breeds were examined. White Leghorns had a higher frequency of the resistance allele (N631) on the Mx1 gene, broilers had a higher frequency of the susceptible allele (S631; Watanabe, 2007). In another study, the resistance allele (N631) was not able to inhibit influenza virus replication in primary chicken embryo fibroblasts from a commercial broiler population (Benfield et al., 2008). Broilers and layers have developed different characteristics of immune systems. Broilers are more specialized in the production of a short-term humoral response (IgM), whereas layers can mount a long-term humoral response (IgG) in combination with a strong cellular-mediated response (Koenen et al., 2002). These might explain different response or results could result from a different type of birds used in the study.

The antiviral activity of the chicken Mx1 gene might also depend on different strains of influenza viruses. A range of influenza A virus strains was tested for murine and human Mx proteins, and remarkable differences among them were found (Dittmann et al., 2008). This was also confirmed in chicken studies. The Mx1 N631 variant alleles had effects on reductions in morbidity, early mortality, viral shedding, and cytokine responses in chicken infected with high pathogenic AIV (H5N2), whereas the results were not reproduced in vitro using a low pathogenic (**LP**) AIV (H5N9) strain (Ewald et al., 2011). The LPAIV strains were used in the current studies, which might contribute the outcome of the study in which no significant associations between AIV replication and Mx1 genotypes were found. It was shown in a recent study that the NP protein of AIV was the main determinant of Mx1 anti-viral sensitivities to influenza virus infections in mice (Zimmermann et al., 2011), which means the variation of viral components could also contribute to the antiviral activities of the Mx protein.

In summary, we developed a very efficient PCR-LP approach using a single PCR reaction to screen the genotypes of G2032A (S631N) of the chicken Mx1 gene. Our results first suggest that there was no significant association between the Mx1 genotype (N631) and AIV replication in chicken both in ovo and in vivo using LPAIV infection, although birds carrying the resistant G2032 (N631) had a tendency of lower virus titers and greater Mx1 gene expression levels than chickens carrying the susceptible A2032 (S631). In addition, we first reported a recombination breakpoint within the Mx1gene in animals, which has laid a solid foundation for further understanding biological function of the Mx1gene in the chicken. Finally, multiple coding region variations of the Mx1 gene in an intercross-derived population suggested great genetic diversity of this gene and its evolutionally not conserved biological function. This has expanded our knowledge of the potential role of the chicken Mx protein on the genetic resistance to AIV in chickens and its potential application in the poultry breeding industry.

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