Influences of MHC Class II Haplotypes on Avian Influenza Traits in Thai Indigenous Chicken

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Natural infections with influenza viruses have been reported in a variety of animal species including human, pigs, horses, sea mammals, mustelids and birds. Occasionally devastating pandemics occur in domestic chickens (broiler and layers) and human. During November 2003 to March 2004 in many countries in Asia, there are outbreaks of H5N1 avian influenza virus, causing of death of infected patients and devastated poultry industry. Some groups of Thai indigenous chickens are survivable recommended as resistance. These traits were related to immunogenetics, especially, the major histocompatibility complex (MHC) class I and class II molecules. The chicken MHC class II was investigated as candidate genes for avian influenza resistance. Seven hundred and thirty Thai indigenous chickens of small holder farms in the rural area of avian influenza outbreaks in the central part of Thailand were used in this study. They were separated into two groups, 340 survivable chickens and 390 dead chickens (resistance and susceptible). Genomic DNA were precipitated from blood samples and feathers. Diluted DNA was amplified to identify MHC haplotype. Data were statistically analyzed by χ^2 analysis to test significant differences of influences of MHC class II haplotypes on avian influenza traits. The results represented ten MHC class II haplotypes, A9, B12, B13, B14, B19, B21, B2, B4, B5, and B6, and included eighteen of their crossbred. The homozygous B21 from these collected samples had 100 percent of survival rate and they were the major survival group. In addition, the heterozygous B21 also showed high survival rate because of co-dominant expression of these genes. In the other way, the homozygous B13 had 100 percent of mortality rate and they were the major group of high mortality rate. These results confirmed that MHC class II haplotypes influenced on avian influenza resistant traits in Thai indigenous chicken. The MHC genes can be used as genetic markers to improve disease resistant traits in chicken.

Key words : avian influenza virus, major histocompatibility complex, thai indigenous chicken

Introduction

The chicken MHC lies on chromosome 16, which is a microchromosome. It was included various linked

multigene families of highly polymorphic genes (Li *et al.*, 1997). It was succeedingly discovered to contain three loci, BF, BL, and BG (Pink *et al.*, 1977), producing class I, II, and IV antigens, which

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expressed on the surface of all nucleated cells, immunological cells, and erythrocytes respectively (Ewert *et al.*, 1980 ; Nordskog *et al.*, 1987). MHC worked as antigen presenting to T lymphocytes as central role of immune system (Abbas *et al.*, 2000). It was first identified by the ability of leukocytes to give strong graft rejection. There is some evidence for different MHC haplotypes having different competence in graft vs. host responses (GVHR), and this was conformed as a mechanism for resistance to Marek's disease ; i.e., less proliferation would decrease the chance of lymphoma development, but may also lead to a general decrease in T cell responses (Pazderka *et al.*, 1975). However, there is no research about association of MHC and avian influenza. It is very important to find candidate genes relating to avian influenza as genetic markers for disease resistant traits improvement.

During November 2003 to March 2004 in many countries in Asia, there were outbreaks of H5N1 avian influenza virus, causing of death of infected patients and devastated poultry industry. Avian influenza outbreaks could be difficult to control and often caused major economic impacts for poultry farmers in affected countries, since mortality rates were high and infected fowl generally might be destroyed in order to prevent the spread of the disease. As a result of ongoing outbreak in Asia, FAO estimated that around 20–25 million birds had been destroyed. For this reason, the local economies of both commercial poultry operations and smallholders were devastated, especially in Thailand, where the industry was heavily reliant on trade. In 2003, poultry exports from Thailand accounted for nearly 7 percent of global poultry meat trade, with an export value of approximately US\$1 billion. Avian influenza outbreak severely decreased frozen poultry meat exportation. The most importance of this outbreak, it caused of death of infected patients in Thailand and Vietnam. Thus, disease resistance was an important economical consideration to poultry industry. During this outbreak, some groups of Thai indigenous chickens were survivable recommended as resistance. This situation indicated that disease resistant traits can be improved genetic selection of genetic markers related to these traits.

Insecurity to disease also might destroy Thai domestic supplies as well as exports of this important food commodity. An interesting alternative ap-

proach to standard disease control methods would be selective breeding to increase disease resistance in livestock. Genetic resistance to disease involved many facets of the body's defense system and their interactions and was extremely complex. Disease resistance research had included measurement of genetic control of disease losses, estimation of heritabilities, and characterization of breed or strain differences. By the reason of production traits genetic improvement made high power of genetic selection for high production traits, the result was down for disease resistant traits. Also, by the way of negative correlations between production traits and disease resistant traits, in addition that disease resistant traits had low heritability, this made difficult to use conventional breeding to improve disease resistant traits. New opportunities to improve our understanding of the genetic nature of disease resistance occur through the recent advances in molecular biology, gene mapping, and immunology and make selection for disease resistance possible in the future. However, genetic considerations included with testing and selection for disease resistance and improved immune responsiveness would need knowledge of the genetic correlations among disease resistance and immune responsiveness and production traits. However, there was a little research in this field.

The current focus was on the genetic makeup of the chicken, specifically the major histocompatibility complex (MHC). Due to the involvement in immune responses, the MHC had a large influence on resistance or susceptibility to disease. This promoted the identification of specific chicken MHC haplotypes for disease resistance. Knowledge of the gene might also suggest improved pharmaceutical or immunological therapies. The objective of the present study was to investigate influences of MHC class II haplotypes on avian influenza traits in Thai indigenous chicken to support the idea of disease resistant traits improvement, animal welfare, safe production, and safe food.

Materials and Methods

Chicken

The chickens used in this research were seven hundred and thirty Thai indigenous chickens in rural area of central part of Thailand in the area of avian influenza outbreak such as Ayuthaya, Kanchanaburi, Nakhon Pathom, Singburi, and Suphanburi prov-

ince, composed of 390 and 340 dead and survived chickens respectively. There was no outbreak of avian influenza in the former time. Chickens never had avian influenza vaccine. They were kept from small holder farms that there were dead and survivable chickens in each farm from avian influenza. About survivable chickens, some were not sick and some were sick, but there were outbreak in every farm of blood collected. Some of sick chickens were died and some were survived. Each farm was random to collect chicken blood for titer testing.

Chicken DNA Extraction

Blood and feather samples were collected from two groups of chickens, survivors and deads from outbreak area. They were used to extract genomic DNA. Extraction of DNA from blood was performed by 0.5 ml of chicken blood collected from the wing vein and kept in micro-tube with EDTA. Blood was treated by lysis buffer, consisted of NaCl, Tris-HCl (pH 8.0), EDTA, SDS, left in 55°C incubator 10 minutes, and added by proteinase K, left in 55°C incubator 2 hours. The samples were treated with the combination of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), mixed, and separated. The upper layer were phenol-extracted, centrifuged, upper layer collected, and added by 95% ethanol, DNA sample were collected in micro-tube, washed by 70% ethanol two times, and left to remove ethanol. The DNA samples were added by TE buffer, consisted of 10 mM Tris and 1 mM EDTA, pH 7.0, and kept at -40°C. DNA extracted from feather was performed by using 50 mg clean individual tip feather. Tip feather was cut into small pieces and soaked in guanidine solution, left in 55°C incubator > 18 hours, and added by proteinase K, left in 55°C incubator 2 hours. The samples were treated with the combination of phenol : chloroform : isoamyl alcohol (25 : 24 : 1), mixed, and separated. The upper layer were phenol-extracted, centrifuged, upper layer collected, precipitate by 2 times volume of 95% ethanol added with 10% of 3 M sodium acetate, and 12000 rpm centrifugation. Precipitant was collected and washed by 70% ethanol two times, and left to remove ethanol (modified from Griffiths and Tlwarl, 1995 ; Sambrook and Russell, 2002). The DNA samples were added by distilled water, and kept at -40°C.

Each DNA sample concentration was measured by the comparison between the concentration of

DNA band of agarose gel and standard concentration. Genomic DNA sample were mixed with steamed distilled water for appropriate concentration (20 ng/ μ l), and kept.

PCR Amplification

Genomic DNA from chickens, extracted by previous method were used to identify MHC haplotype by PCR of microsatellite marker, LEI0258. DNA samples were amplified by using forward and reverse primers (5'-CACGCAGCAGAACTTGGTAAGG-3' and 5'-AGCTGTGCTCAGTCCTCAGTGC-3') (McConnell *et al.*, 1999). This microsatellite was tetranucleotide microsatellite near BL β I on micro-chromosome 16. Each 15 μ l reaction contained 20 ng of DNA, 0.5 μ M of each primer, 1.9 mM MgCl₂, 0.2 mM of each dNTP and 1 unit of *Taq* DNA polymerase in the supplied buffer (final concentration : 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl pH 9.0, 0.01% (w/v) Tween). PCR amplification of DNA was performed using Touchdown PCR in a PTC-100™ Programmable Thermal Controller using the reaction profile of initial denaturation of 94°C 3 min, denaturation of 94°C 0.45 min, annealing 59°C 0.45 min, temperature increment -1°C/cycle, extension of 72°C 1 min, for 5 cycle, denaturation of 94°C 0.45 min, annealing 54°C 0.45 min, extension of 72°C 1 min, for 25 cycle, and final extension of 72°C 10 min. Each group of genomic DNA separated by LEI0258 typing were partial sequencing of BL β I exon2 PCR (Forward primer 5' GTGCCCCGACGCTTCTTC 3', Reverse primer 5' TCCTCTGCACCGTGAAGG 3') (Goto *et al.*, 2002). Each 20 μ l reaction contained 20 ng of DNA, 0.5 μ M of each primer, 1.9 mM MgCl₂, 0.2 mM of each dNTP and 1 unit of *Taq* DNA polymerase in the supplied buffer. PCR amplification of DNA was performed in a PTC-100™ Programmable Thermal Controller using the reaction profile of initial denaturation of 94°C 3 min, denaturation of 94°C 0.45 min, annealing 54°C 0.45 min, extension of 72°C 1 min, for 35 cycle, and final extension of 72°C 10 min. Aliquots of the PCR reactions were checked for robustness using 2% agarose gel electrophoresis before proceeding to automated sequencing.

Each group from LEI0258 typing was standardized by partial sequencing of BL β genes to identify MHC haplotype.

Estimation of Influences of MHC Haplotypes on Avian Influenza

Data were statistically analyzed by χ^2 analysis to test significant differences of influences of MHC class II haplotypes on avian influenza traits in Thai indigenous chicken.

Results and Discussion

Situation of Avian Influenza Outbreaks in Thailand

Avian influenza was caused by avian influenza virus type A, capsulated RNA virus of Orthomyxoviridae family. It has 2 specific surface antigens, haemagglutinin (H, H1-H15) and neuraminidase (N, N1-N9). During November 2003 to March 2004 in many country in Asia, such as Cambodia, China, Hong Kong, Indonesia, Japan, Korea, Laos, Vietnam, and Thailand, there were outbreaks of avian influenza, especially in Vietnam and Thailand, strain of H5N1, causing of death of infected patients and devastated poultry industry. The H5N1 strain had a different genetic sequence and therefore is believed to come from a different source. It is violent pathogenic Avian Influenza. In Thailand, there are ongoing outbreaks of avian influenza. It devastated to local economies and to both commercial poultry operations and smallholders. Chicken prices are down slightly this year in the EU. While a ban on Thai chicken will leave a deficit in the market for proceed white meat, it is likely that the Brazilians, a competitive producer of similar type of product, will move to supply the gap. In the Japanese market, domestic poultry supplies are dropping as avian influenza infected flocks are disposed of and imports are banned from Thailand (Thailand accounts for thirty percent of Japanese poultry imports). This will push prices of all meats higher. Interestingly, some of Thai indigenous chickens survived from these outbreaks. It meant that these chickens resisted to avian influenza. They were suitable to use as genetic resources for genetic improvement of virulent disease resistant traits in poultry industry in Thailand.

MHC Class II Haplotypes Genotyping

MHC class II haplotypes genotyping was used the previous method. The results represented ten MHC class II haplotypes, A9, B12, B13, B14, B19, B21, B2, B4, B5, and B6, and included eighteen of their crossbred. Haplotype of A9 of MHC class II was haplotype in broiler chicken found by Li *et al.*

(1999), different from standard haplotypes in White Leghorn chicken. Standard haplotype in White Leghorn chicken preceded with the letter 'B' and followed by numeric. Many scientists suggested that nucleotide sequencing of MHC haplotypes from different breeds were different, because of cross-reaction of serological reagent of White Leghorn using in broiler chicken (Li *et al.*, 1999). B2–B21 MHC class II haplotypes were identity to standard haplotypes of White Leghorn chicken.

Influences of MHC Class II Gene Polymorphisms to Avian Influenza Traits

The MHC haplotypes were identified in Thai indigenous chickens of small holder farms in the rural area of avian influenza outbreaks in the central part of Thailand represented 28 groups of homozygous and heterozygous haplotypes (Table 1).

The B21 haplotype in MHC class II was the strongest resistant haplotype. The haplotype of B21 survived 100 percents in class I (data not shown). The B12 haplotype was found in dead chickens, recommended as susceptible group. The B12 haplotype in many reseaches were the same case in as this study. It was susceptible to disease (not all disease) (Bacon, 1987). In MHC class II haplotypes, similarly to MHC class I, homozygous B21 haplotype chicken survived 100 percents and they were major group, also to their crossbred with other haplotypes were high survival percentage, because of effected expression as co-dominant of this gene. The B14 homozygous haplotypes and their heterozygotes were high survival. In addition, A9 haplotype was 90 percents survival rate. The sequences of B21, B14, and A9 haplotype at exon2 of BL β were almost similar. This region of gene produced protein as pathogen binding site. This region was highly polymorphic and specific to pathogen in each haplotypes. The B13 haplotype were 100 percents of mortality rate, and they were the major group of dead chickens. The same as B19 haplotype, they were 100 percents dead. However, their heterozygous haplotypes were not 100 percent survived, or 100 percent dead, because the effect of co-dominant gene expression.

Thai indigenous chickens of small holder farms in the rural area were raised according to natural environment. They were selected by natural selection. The strong chickens were alive and reproductive. The susceptible chickens were culled by nature

Table 1. Influences of MHC class II gene polymorphisms to avian influenza traits

Haplotypes ^{1/}	Number of chicken ^{2/}	% survival ^{3/}
LHK		
A9A9	31	90.32 ^a
B12B12	39	0.00 ^c
B12B13	4	0.00 ^c
B12B2	24	66.67 ^b
B13B13	71	0.00 ^c
B13B2	6	0.00 ^c
B14B14	8	100.00 ^a
B14B19	4	0.00 ^c
B14B2	3	100.00 ^a
B19B19	5	0.00 ^c
B21B21	74	100.00 ^a
B21B4	5	20.00 ^c
B2B2	6	66.67 ^b
B2B21	10	90.00 ^a
B2B5	16	43.75 ^b
B4B4	4	50.00 ^b
B5B5	3	66.67 ^b
PHD		
A9A9	31	90.32 ^a
B12B12	92	0.00 ^c
B12B13	4	0.00 ^c
B12B2	24	66.67 ^b
B13B13	71	0.00 ^d
B13B2	6	0.00 ^d
B14B14	2	100.00 ^a
B14B19	4	0.00 ^d
B14B2	3	100.00 ^a
B19B19	5	0.00 ^d
B21B21	103	100.00 ^a
B21B4	5	20.00 ^d
B2B2	6	66.67 ^b
B2B21	7	85.71 ^a
B2B5	16	43.75 ^c
B4B4	4	50.00 ^b
B5B5	3	66.67 ^b

^{1/}LHK = Leung-Hahng-Kow chicken, PHD = Pradoo-Hahng-Dam

^{2/}Data not shown for minor group chicken.

^{3/}The different letter in same class of MHC means highly significant difference ($P < 0.01$).

passed through many generations, dead, and extinct.

Further experiment, combination between MHC class I and II affecting on avian influenza will be apparently detected. The various combination among haplotypes showed various levels of disease resistance. The regions of BL β I exon2 of MHC class II haplotypes were specific to pathogen, pathogen binding site, so MHC class II haplotypes were highly polymorphism in disease resistant traits (Livant *et al.*, 2001).

After the outbreaks, many of chickens including survived Thai indigenous chickens, were destroyed to eradicate avian influenza. It is suitable to short

term controlling. In long term controlling, it is better to improve disease resistant traits in poultry by using candidate genes of disease resistance as genetic marker associating with conventional breeding. Disease resistant trait improvement can't process in only conventional breeding according to negative correlation between production traits and disease resistant traits. High intensity of selection of production traits compresses disease resistant traits (Rothschild, 1989). In conventional breeding, step of progeny test, it can't challenge pathogens to select sire or dam. Therefore, genetic markers of disease resistant genes such as MHC genes, are suitable to improve disease resistant traits.

The MHC class II genes associated to avian influenza trait in Thai indigenous chickens. There are polymorphisms of these genes. The MHC genes can be used as genetic markers to improve disease resistant traits in chickens (indigenous chicken, broiler, and layers) for disease prevention. Finally, poultry industry has high standards in the world for raising healthy livestock and providing consumers with safe products.

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