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Identification of Avian Influenza Genetic Resistance Gene Marker in Chickens

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Abstract

The Mx gene is a potential candidate as a genetic resistance marker to the avian influenza virus in chickens. The purpose of this study is to identify the potential of Mx gene as a marker of resistance to avian influenza viruses in various breeds of chicken. The study used an exploratory design using three common chicken breeds. The Kampong chicken, the Hy-Line Brown laying hen strain, and the White Leghorn broiler strain were collected from the local farms, and blood samples were drawn from each. The DNA of each sample was amplified by a pair of Mx gene primers (NE-F2/F and NE-R2/R). The polymerase chain reaction (PCR) product of Mx gene fragment was cut using restriction enzymes *Rsa*I to determine the genotype of the Mx gene. The PCR-RFLP analysis in this study showed that 100% of the broilers were GG genotype. In laying hens, 80% were AG genotype, 10% GG genotype, and 10% of the sample was not successfully analyzed. In the Kampong chicken, 68.4% were GG genotype, and 31.6% were AG genotype. The results showed that DNA mutation identification can be applied to determine the genotype chicken against virus. The genotypes of Mx genes in chickens indicates avian influenza virus resistance; whereas, the chicken phenotype does not suggest their level of resistance.

Abstrak

Identifikasi Gen Penanda Resistensi terhadap Avian Influenza pada Ayam. Gen Mx berpotensi sebagai penanda gen resistensi terhadap virus avian influenza pada ayam. Tujuan penelitian ini adalah untuk mengidentifikasi potensi gen Mx sebagai penanda resistensi terhadap virus avian influenza pada berbagai jenis ayam. Penelitian ini menggunakan desain eksploratif pada 3 jenis ayam yaitu ayam kampung, ayam petelur dan ayam pedaging yang diperoleh dari peternakan tradisional. DNA dari masing-masing sampel diamplifikasi dengan sepasang primer spesifik untuk gen Mx (NE-F2/F dan NE-R2/R). Produk PCR dipotong menggunakan enzim restriksi *Rsa*I untuk menentukan genotipe gen Mx. Analisis PCR-RFLP dalam penelitian ini menunjukkan bahwa semua (100%) ayam pedaging bergenotipe GG. Pada ayam petelur, ditemukan bahwa 8 ekor (80%) bergenotipe AG, 1 ekor (10%) bergenotipe GG dan 1 ekor (10%) tidak berhasil dianalisis. Pada ayam kampung, 13 ekor (68,4%) bergenotipe GG dan 6 ekor (31.6%) bergenotipe AG. Hasil penelitian menunjukkan bahwa identifikasi mutasi DNA dapat diterapkan untuk menentukan genotipe ayam terhadap virus avian influenza. Genotipe berdasarkan gen Mx (GG, AG, dan AA) menunjukkan tingkat resistensi ayam terhadap infeksi virus avian influenza, namun fenotipe ayam tidak menunjukkan karakter resistensinya terhadap virus avian influenza.

Keywords: Avian influenza virus; chicken species; genetic marker; Mx gene

Introduction

In recent years, the attention of the world, particularly Indonesia, has been focused on the outbreak of the avian influenza virus (AIV) subtype H5N1, better known as bird flu. Outbreaks of avian flu have caused the deaths of birds and even humans. The increasing number of zoonotic transmissions to humans is increasing the potential for a pandemic [1]; however, human-to-human transmission of AIV subtype H5N1 has not been reported [2-4], and to date, AIV transmission to humans is still considered to occur directly from poultry (chickens) or AIV-contaminated environments [1]. Pandemics occur when a new influenza virus subtype crosses the

barrier between avian and mammalian hosts, including humans. AIV subtype H5N1 has the potential to cause a human influenza pandemic through two mechanisms: the first one is triggering of re-assortment in AIV subtype H5N1 and human influenza strains (H1N1) which raises the AIV transmission from human to human; and the direct mutation of AIV subtype H5N1, which leads to transmission from human to human [5].

Thus far, high pathogenic avian influenza (HPAI) virus outbreaks are primarily caused by influenza virus subtypes H5 and H7. The global distribution of the virus is caused by poultry and poultry product trading, the mobility of people (tourists and refugees), as well as bird migration [3, 6-7]. Analysis of the spread of AIV subtype H5N1 in Asia shows that 9 out of 21 virus outbreaks in Asian countries were initiated through poultry or poultry products. Migratory birds also played a role in the spread and introduction of AIV subtype H5N1 to 3 out of 21 nations in Asia. Meanwhile in Europe, AIV outbreaks in 20 out of 23 countries were caused by migratory birds. In Africa, 2 out of 8 countries experienced outbreaks of AIV subtype H5N1 in the poultry market, and outbreaks in 3 out of 8 countries were a result of bird migration [8]. Studies found that the AIV subtype H5N1 isolated from waterfowl forms three separate branches: the Indonesia cluster and two branching clusters in the Asia group. The introduction of AIV subtype H5N1 in Indonesia has occurred more than once [9], where the virus was possibly spread by wild migratory birds [1,6] as well as through trading and transport of poultry and poultry products between locations [10]. By the end of June 2006, 31 of 33 provinces in Indonesia had confirmed outbreaks of H5N1 in poultry, resulting in more than 16 million poultry deaths from sickness and culling [11].

In addition to causing casualties, the outbreak of bird flu also has a detrimental impact on economic sectors, including farmers and the poultry industry [12]. Since news of the virus has spread, many traditional markets have seen a decline in demand for poultry, especially meat, to 60% throughout many regions, particularly in Semarang, Central Java. To contain the presence of the virus, the government has implemented a complex disinfectant spraying method in various markets [13]. In November 2008, the Semarang government announced that bird flu in Semarang was categorized as an “extraordinary event,” or *Kejadian Luar Biasa (KLB)*. This action was taken after an anti-dsDNA blood sample test on a 15-year-old resident of the Gayamsari District showed a positive result of AIV subtype H5N1, the first occurrence in Semarang of bird flu infection. After the announcement, AIV infection prevention and control actions were taken by both society and government. Bird flu prevention and control methods included the application of biosecurity and vaccination, but because the avian influenza virus mutates very rapidly, vaccination is sometimes ineffective. Implementation of biosecurity and vaccination in commercial breeding farm enterprises does not cause any problems; however, in small poultry husbandries, especially local farms, the animals are mostly domesticated wild birds, making the application of biosecurity and vaccination methods difficult. In fact, the majority (80%) of local chicken farms in Indonesia are small farmers. Therefore, it is necessary to provide an alternative local poultry system to select the animals that are resistant to avian flu.

The genetic approach can successfully attain the goal of AIV resistant chickens. The development of science and technology facilitates a highly efficient genetic selection that is faster and more accurate than the application of conventional selection methods using morphological characteristics-based, biosecurity and vaccination methods [14]. The use of the gene as a marker candidate is appropriate because it is a blueprint of living things, and the genome research results are guaranteed for its accuracy and is listed on the database by accession number in the gene bank. By using this method, the determination of the gene can be controlled, and the application of molecular selection for poultry to get the desirable criteria will be more easily achieved. One potential gene marker is the Mx gene. The Mx gene was first identified in 1980 and is involved in the defense mechanism against avian influenza virus infection in birds [15]. The Mx gene was found in chickens during an AIV outbreak in domestic poultry. In chickens, the Mx gene is located on chromosome 1 at the 20,767 bp fragment. The gene consists of 13 exons, comprising 2,115 bp coding regions with 705 amino acids. The resistance to AIV presents in exon 13, nucleotide number 1,892 at a single mutated point [16]. Point mutation is a mutation transition from adenine (A) to guanine (G). This mutation causes a shift in codon 631, changing the asparagine (AAC/AAU) to serine (AGC/AGU). Chickens with asparagine on its Mx gene encoding indicates that the chicken is resistant to the avian influenza virus and is referred to as the Mx^+ gene. When the mutation occurs with serine, this polymorphism causes susceptibility to the avian influenza virus subtype H5N1 and is referred to as Mx^- [16,17].

Ommeh *et al*. [18] had studied the genotyping of native chicken populations from Asia, Europe, Africa, and Indonesia. They examined the Indonesian native chickens such as Kedu chicken, Sentul chicken, Kate chicken, Gaok chicken, Cemani chicken, Wareng chicken, and Cianjur chicken. However, the genotyping of the Kampong chicken was not studied; therefore, this research on the Kampong chicken will contribute to current genotype information. This information could be useful for house scale breeders to select the AIV- resistant chicken strain. The Mx gene could be the best molecular

marker for the selection of qualified chicken breeds to achieve effective breeding management.

Materials and Methods

Materials. Blood samples were collected from the Kampong chicken, the Hy-Line Brown laying hen strain, and the White Leghorn broiler strain. Various chemical reagents for genomic DNA extraction were bought from Merck (Germany), Vivantis (Malaysia), and Roche (Switzerland). Agarose used for DNA quantification and PCR-RFLP analysis were purchased from Merck (Germany) and MetaPhor™ Fisher Scientific (USA). Primer pairs for PCR reaction were obtained from AlphaDNA (Canada). PCR reagents and restriction enzyme *Rsa*I were obtained from Thermo Fisher Scientific (USA).

Sampling method. Blood was collected through the brachial veins from Ten Hy-Line Brown egg-layers, ten White Leghorn broilers, and nineteen Kampong chickens (16 males and 3 females). The samples were preserved in EDTA and were then taken to the laboratory for further processing.

Genomic DNA extraction and DNA quantification. Genomic DNA was extracted from whole blood using the salting out method [19]. DNA purification was performed using the NucleoSpin Blood kit (Macherey-Nagel, Germany). Subsequently, the DNA quality and quantity were measured using gel electrophoresis and spectrophotometry analysis, respectively. DNA electrophoresis was performed on agarose 1% (w/v), which was prepared in 1× TAE buffer; DNA staining was done using Ethidium Bromide. The DNA purity measurement using spectrophotometry analysis was accomplished to calculate the ratio of OD_{260} to OD_{280} of each DNA sample.

Mx Gene Amplification and PCR-RFLP for geno type identification. The PCR-RFLP method was used to analyze the genotype of nucleotide position 1892 in exon 13 of the Mx gene coding sequence. The primer sequences that amplified approximately 100 bp fragments were done according to the method used by Seyama *et al.* [20]. The PCR mix comprised 0.1 µM forward primer, 0.1 µM reverse primer, 50 ng DNA template, PCR master mix, and ultrapure water. The PCR was performed using an initial denaturation at 94 °C for 5 min, followed by 35 cycles of 60 sec at 94 °C, annealing temperature for 60 sec at 60 °C, and 72 °C for 60 sec, and the final extension at 72 °C for 5 min (PeqSTAR thermocycler, Peqlab, GmbH., Germany). The PCR product was analyzed by electrophoresis on 2% agarose gel in 1x TBE buffer and stained with EtBr. The restriction enzyme *Rsa*I with a recognition sequence of 5'GT↓AC3' was employed to cut the fragment where an allele G is located in the coding sequence [21,22]. To identify the genotype of the Mx gene, 10 µl amplicons were cut by the *Rsa*I restriction enzyme (1U/µg DNA) for 6-8 hours at 37 °C, following manufacturer's instruction. The digested fragments were visualized by 3% superfine resolution (SFR) Meta Phor™ agarose gel in 1× TBE buffer [23].

Data analysis. The results of PCR-RFLP was analyzed based on the clue of avian influenza virus resistance found in amino acid 631 in exon 13, which was triggered by a mutation of transition base (single/point mutation) GC into AT. The mutation caused the transformation of serine to asparagine that resulted in waterfowl resistance to the avian influenza virus (Mx^{++}); whereas, the serine amino acid indicated waterfowl susceptibility to the bird flu virus (Mx^{--}). An A/A genotype with 100 bp product of enzyme restriction showed a homozygous resistant Mx^{++} allelic gene; whereas, two bands with 100 bp and 73 bp in length showed an A/G heterozygous Mx^{+-} allelic gene, and one band with 73 bp showed a G/G homozygous sensitive Mx^{--} allelic gene [23,24].

Results and Discussion

All chicken DNA samples generated 100 bp PCR products, as illustrated in Figure 1. Several study results indicate that the Mx gene is a potential genetic marker [20-23,25-27]. According to Seyama *et al.* [20], NE primers are widely used in Mx gene identification studies in chickens and waterfowl. Moreover, the PCR-RFLP method can be used to determine the Mx gene polymorphism in chickens [20,21,23,26,28]. The polymorphism of G/A at position 2032 of the chicken Mx gene resulted in a possible Mx protein at amino acid number 631 from serine (S) to Asparagine (N). In fact, one non-synonym mutation (S631N) is reported to be associated with antiviral capability against avian influenza virus infection *in vitro* [21]. The amino acid asparagine at number 631 indicates avian influenza virus resistance in chickens; whereas, the amino acid serine at number 631 indicates virus susceptibility in chickens. The S631N mutation can be detected using PCR-RFLP [20,21,23,26,28]. The PCR-RFLP method can also identify Mx gene polymorphism in pigs due to VSV infection [29]. Mx gene fragments were cut with *Rsa*I enzyme at 5'GT AC3' to determine the Mx gene polymorphism characteristic [21,22]. With PCR-RFLP, three variations of different genotypes of Mx gene were

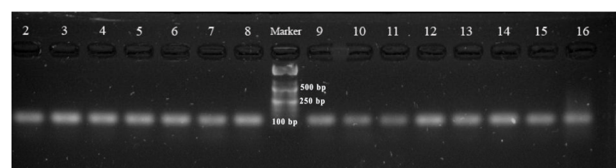

Figure 1. Results of Mx Gene Amplification in Chicken DNA Samples Showing 100 bp PCR Products

identified: the resistant gene (Mx^{++} represents genotype AA or allele A); the resistant/sensitive gene (gene Mx^{+-} represents genotype AG or allele R); and the sensitive gene (Mx^{--} represents genotype GG or allele G).

Genotype AA shows a 100 bp DNA fragment, which indicates Mx^{++}; whereas, genotype AG indicates two DNA fragments of 100 bp, and 73 bp indicates Mx^{+-}. Genotype GG shows DNA fragments of 73 bp, and 27 bp shows Mx^{--} [20] (Figure 2). The research by Sartika *et al.* [23] revealed that the local chickens from 12 Asian countries have Mx^{++} and Mx^{--}. The chicken population has a different phenotype regarding resistance to the avian influenza virus, i.e. resistant and sensitive. The frequency of the Mx^{++} gene is higher than the Mx^{--} gene in native chickens in several countries, including Indonesia. The PCR-RFLP results of 485 chicken samples from 15 locations in Indonesia showed that the Cemani chicken was the most resistant compared to other chicken species [25].

In this research, PCR-RFLP results showed a variation of RFLP products on different strains of chickens (Table 1). All broilers produced the 73 bp DNA fragment showing sensitive or genotype GG, while egg-layers dominated as genotype AG (80%). The backyard chickens showed two variations of Mx gene genotypes: 68.4% were GG, and 31.6% were AG. PCR-RFLP analysis suggests that all broilers are GG genotype. In laying hens, 8 were AG genotype, 1 was GG genotype, and 1 sample was not successfully analyzed. In the Kampong chickens, 13 were GG genotype, and 6 were AG genotype. The results indicate that mutation identification is

Figure 2. PCR-RFLP Products of Chicken Mx Genes Cleaved by *Rsa*I in 3% MetaPhor™ Agarose Analysis. Samples 1-3: Broilers; 4-8: Laying Hens; 9-16: Kampong Chickens

Table 1. The Results of Chicken Breed Genotypes based on PCR-RFLP Product

Chicken breeds	Genotype GG (sensitive)	Genotype AG (heterozygote)	Genotype AA (Resistant)
White Leghorn Broilers	10 (100%)	0	0
Hy-Line Brown Egg-layers	1 (10%)	8 (80%)	0
Kampong	13(68.4%)	6 (31.6%)	0
Male	10 (62.5%)	6 (37.5%)	0
Female	3 (100%)	0	0

necessary because the GG, AG, and AA genotypes show the chickens' level of susceptibility to the avian influenza virus. However, the chickens' phenotype themselves do not present the specific characteristic of virus. This fact shows that chicken characteristics and chicken species do not indicate the level of resistance to the avian influenza virus or their actual biological significance. Research by Susanti *et al.* [30] shows that all waterfowl Mx^{--} monomorphic genes present serine at amino acid number 631, or showing the GG genotype. The samples also indicate that waterfowl are sensitive to the avian influenza virus infection, revealing as well that the Mx protein in waterfowl shows no increase in antiviral activity due to viral infection. The research of Li and Lu [17] showed that the Mx protein in all types of wild duck has serine (AGC/AGU) at amino acid number 631. Likewise, the wild ducks in the Cagar Alam Pulau Dua region of Indonesia showed no variation of Mx genes, all of which have the genotype GG [26]. These data support the theory that antiviral activity of the Mx protein varies between organisms. These variations are affected by the mechanism factors of host and virus resistance or sensitivity to infectious diseases. Research by Dillon & Rustandler [31] shows the Mx gene variation in five species of ducks in Alaska (*Anas creccacarolinensis*, *A. americana*, *A. platyrhynchos*, *A. acuta,* and *A. Clypeata*). They found 16 variations of unique protein sequences in the Mx gene. The results of these analyses suggest that some chicken and waterfowl Mx alleles may have antiviral activity, and this putative activity appears to be conferred by the change from serine to asparagine at amino acid 631 and is often referred to as the resistance allele [23]. Moreover, the survey shows that native chickens have a higher frequency of resistant alleles than broilers [16,20]. As mentioned by Clark [32], the Mx gene variation analysis approach as conducted among different chicken species, while often a first step in understanding how a specific sequence is associated with a trait of interest, does not account for the context of the variants.

The study by Fulton *et al.* [33] suggests that the Mx protein is large and complex, with multiple functional domains in which each domain plays a role in the Mx oligomer and its last function in the host. The variation of its SNP and haplotypes in the cell is key to understanding the role Mx plays in the interferon-mediated response to viral infections in chickens. Furthermore, the Mx gene is a member of the guanine-3 phosphokinase gene family, while its expression is induced by interferons [34]. At the cellular level, Mx genes are expressed into many Mx proteins that contribute to chicken species' resistance to different diseases. Single base mutations at the Mx gene can trigger the resistance potency in animals. In chickens, the Mx gene is located on chromosome 1, and the resistance level of avian influenza virus presents in exon 13, at codon 631

that codes asparagine (AAC/AAU), or the Mx++ gene. Data on the Mx gene obtained from infected animals suggest that chickens have a weak level of resistance to avian influenza virus; whereas, a high level of resistance are found in domestic waterfowl [35].

Conclusion

The PCR-RFLP analysis in this study showed that all White Leghorn broiler strain were GG genotype, 80% of the Hy-Line Brown laying hens were AG genotype, 10% GG genotype, and 10% of the sample was not successfully analyzed. In the Kampong chicken, 68.4% were GG genotype, and 31.6% were AG genotype. The genotypes of Mx genes in chickens indicates avian influenza virus resistance; whereas, the chicken phenotype does not suggest their level of resistance. Genotype analysis of antiviral activity against the avian influenza virus infection based on the Mx gene is needed to proceed with the analysis of their phenotype characteristics.

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