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Identification of Gene Resistance to *Avian Influenza* Virus (Mx Gene) among Wild Waterbirds

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Abstract

The *Mx* gene is an antiviral gene used to determine the resistance or the susceptibility to different types of viruses, including the Avian Influenza (AI) virus subtype H5N1. The AI virus subtype H5N1 infection in chickens causes *Mx* gene polymorphism. The Mx^+ gene shows resistant to the AI virus subtype H5N1, whereas the Mx^-gene shows signs of susceptible. The objective of this research was to detect the *Mx* gene in wild aquatic birds using the Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) method with the primer pairs F2 and NE-R2/R and the RsaI restriction enzyme. DNA samples were obtained from eight species of wild waterbirds with positive and negative exposure to the AI virus subtype H5N1. DNA amplification results showed that the *Mx* gene in wild aquatic birds is found in a 100 bp fragment, which is the same as the Mx gene found in chickens. However, unlike chickens, the *Mx* gene in wild aquatic birds did not show any polymorphism. This study proves that *Mx*- based resistance to AI virus subtype H5N1 in different in wild birds than in chickens.

Abstract

Identifikasi Gen Mengatur Resistensi terhadap Virus AI (Gen Mx) pada Burung Air Liar Penetap di Cagar Alam Pulau Dua Serang, Provinsi Banten. Gen *Mx* merupakan gen antiviral yang berperan menentukan sifat resisten atau rentan terhadap berbagai jenis virus, termasuk virus AI subtipe H5N1. Infeksi virus AI subtipe H5N1 pada ayam mengakibatkan polimorfisme gen *Mx*. Gen Mx^+ menunjukkan sifat resisten sedangkan gen Mx^- menunjukan sifat rentan. terhadap virus AI subtipe H5N1. Tujuan penelitian adalah membuktikan bahwa gen Mx terdapat pada burung air liar dan terdapat polimorfisme gen *Mx* pada burung air liar akibat paparan virus AI subtipe H5N1. Metode deteksi gen Mx menggunakan PCR-RFLP dengan sepasang primer NE-F2 dan NE-R2/R dan enzim pemotongan *RsaI*. Sampel DNA burung air liar berasal dari 8 jenis burung air liar yang positif dan negatif terhadap paparan virus AI subtipe H5N1. Hasil amplifikasi DNA menunjukkan bahwa gen *Mx* pada burung air liar dijumpai pada panjang fragmen 100bp, sama seperti gen *Mx* yang terdapat ayam. Akan tetapi gen *Mx* pada burung air liar tidak menunjukan adanya polimorfisme seperti yang dijumpai pada ayam. Penelitian ini membuktikan bahwa keberadaan gen *Mx* pada burung air liar dalam mempengaruhi mekanisme resistensi terhadap paparan virus AI subtipe H5N1 berbeda dengan yang terdapat pada ayam.

Keywords: Mx gene, wild waterbirds, exposure, AI virus subtype H5N1, resistance

1. Introduction

Every organism has a natural ability to defend itself against exposure to microorganisms that invade its body. The defense mechanism is associated with the ability of the innate immune system to detect microorganisms and to produce an appropriate response. Innate immunity is an immune response that is directly produced by the body at the time microorganisms invade the host body. This self-defense system is capable of causing organisms to be resistant or sensitive to invasive microorganisms [1].

The resistance of an organism is associated with its ability to defend itself against attack by microorganisms (viruses, bacteria, fungi). The mechanism of resistance is closely related to the body's immune system, which detects foreign microorganisms or antigens contained in the microorganisms [1].

The *Mx* protein is involved in the control of the immune system, and it is known to inhibit the replication of various viruses. The specific function of the *Mx* protein in defense against viruses is the result of a direct interaction between the carboxyl terminus of the *Mx* protein of specific species of pathogenic viruses [2].

Mx proteins in various organisms are located in different tissues and play various antiviral roles in the cell. The Mx1 protein in the nuclei of primary rat cells inhibits the replication of the orthomyxovirus (including the influenza virus), and the *Mx*2 protein in the cytoplasm is the main inhibitor of the somatic vesicular virus (VSV). In humans, *Mx*A confers resistance to a very broad spectrum of viruses, including orthomyxovirus (also influenza), rhabdovirus, bunyavirus, and paramyxovirus [3].

The *Mx* gene forms an Mx antiviral protein, specifically for influenza, in a number of animals. This is the only known myvovirus-resistant gene [4]. In several organisms, the *Mx* gene plays a role in defense against the AI virus. The presence of this gene also determines whether the organism is resistant or susceptible to viral invasion because it has elements that contribute directly to forming the antivirus and the response to invasion by the AI virus [5-7].

In cattle and dogs, the *Mx*1 and *Mx*2 genes act as specific antiviral agents against VSV [8]. In pigs, the Mx1 gene sequence prevents against VSV attack [9]. The *Mx* gene in salmon also forms *Mx* protein, which resist invasion by the infectious pancreatic necrosis virus and the infectious viral salmon anemia virus [10].

The *Mx* gene was first identified in fowl in 1980 when research on *Mx* genes revealed host defense mechanisms against influenza virus infections [11]. The *Mx* gene was later found in chickens during the outbreak of the AI virus in domestic fowl. In chickens, the *Mx* gene is located in chromosome 1 in a 20.767 bp fragment. It consists of 13 exons, with as many as 2.115 bp coding regions and 705 amino acids. Resistance against the AI virus was found at exon 13, nucleotide number 1.892 where it undergoes alkaline transition mutation (single mutation) [12].

The point mutaton involves a transitional substitution mutation of an alkaline nucleotide base, purine adenine (A), to an alkaline nucleotide base, purine guanine (G). Mutations in these *Mx* genes cause triple codon changes to 631, which mutates the amino acid asparagine (AAC/AAU) to serine (AGC/AGU). The presence of the amino acid asparagine at nucleotide 1.892 indicates that chickens have resistance to AI marked by the Mx^+ gene. If there is a mutation of the amino acid asparagine to serine, the resulting polymorphism pf the *Mx* gene will cause to be susceptible to AI virus subtype H5N1 and AI virus attacks [12-13]. This is what causes polymorphism of the chicken *Mx* gene in the AI virus subtype H5N1.

Research carried out on a number of local chickens originating from 12 countries in Asia showed that all the local chicken population has Mx^+ and Mx^- genes. However, there are differences in the ability of each fowl to resist AI attacks. These birds show two types of defense mechanisms, which make them either resistant or susceptible to the AI virus. Local fowl in a number of countries, including Indonesia, show Mx^+ gene frequencies that are greater than those of Mx^- genes, with Mx^+ gene frequencies of 63% and Mx^- frequencies of 37% reported in Indonesian native chickens. Countries vulnerable to AI have Mx^-gene frequencies greater than 50%, namely Korea, Taiwan, and Sri Lanka [7].

A Polymerase Chain Reaction-Restriction Fragment Length Polymorphism (PCR-RFLP) study of 485 chickens from 15 local strains in Indonesia that survived an AI outbreak showed that the Cemani chicken strain (allele A frequency of 0.89%) has the highest resistance to bird flu virus compared with the other chicken strains. The Kapas chicken (allele A frequency only 0.35%) has the lowest resistance. The results showed that 88% of the Cemani chicken population studied had protein resistance to the AI virus, resulting in a resistance response that is higher than the other strains of chickens [14].

Research on the presence of the *Mx* gene in resident wild waterbirds, especially those from Indonesia, has never been reported. This research aims to identify the Mx gene in resident wild waterbirds on the "Cagar Alam" Pulau Dua (CAPD) or Pulau Dua Nature Reserve and to analyze whether the *Mx* gene polymorphism is caused by exposure to the AI virus in these birds.

2. Methods

Extraction, purification, and DNA electrophoresis. Extraction of DNA from blood samples of resident wild waterbirds was carried out according to [14]. The results of DNA isolation were visualized by electrophoresis on 2% agarose gel in buffer TBE at 100 V 50 mA for 50 min. Visualization of the DNA was also carried out using 12% vertical polyacrylamide gel electrophoresis (PAGE) for 4 hours at 160 V with silver staining [14].

PCR-RFLP analysis. Amplification to detect a single nucleotide polymorphism at nucleotide position 1.892 of the Mx gene DNA sequence located at exon 13 (substitution of amino acid at position 631 of the Mx protein) was performed using the PCR-RFLP method developed by Seyama [19]. The primers used were forward NE-F2 (5'-AGAGGAATCTGATTGC

TCAGGCGTGTA-3') and reverse NE-R2/R (5'-CAGAGGAATCTGATTGC TCAGGCGAATA-3').

PCR amplification was performed on the Applied Biosystem GeneAmp PCR System 9700. The PCR conditions were: predenaturation at 94 °C (5 min), denaturation at 94 °C (1 min), annealing at 61 °C (1 min), elongation at 72°C (1 min), 35 amplification cycles, and a final extension at 72 °C (5 min) [20]. The PCR reaction mixture (30 µl) contained: 1 µl DNA sample, 1 µl forward primer, 1 µl reverse primer, 4 µl $MgCl_2$, 0.6 µl dNTP; 3 µl 10x PCR buffer, and 0.25 µl ml Taq-polymerase.

Successfully amplified DNA fragments were cut with RsaI restriction enzyme, an enzyme that cuts DNA molecules at specific places to obtain a structure base of GT↓AC. The results of the PCR-RFLP cut fragments were checked with 12% PAGE for 4 hours at 160 V, DNA were visualized with silver staining [18].

3. Results and Discussion

A total of 294 samples were successfully obtained from 264 waterbirds, 10 ducks, 10 muscovy, and 10 chickens. The 264 samples of waterbirds consisted of eight species of wild waterbirds from 12 species resident on the CAPD. The eight species included heron (*Ardea* sp.), cormorant (*Phalacrocorax* sp.), great egret (*Casmerodius albus*), intermediate egret (*Egretta intermedia*), little egret (*E. garzetta*), cattle egret (*Bubulcus ibis*), Javan pond heron (*Ardeola speciosa*), and black-crowned night heron (*Nycticorax nycticorax*).

The PCR analysis of the DNA from the eight species of resident wild waterbirds, one chicken, one duck and one muscovy from the CAPD using the NE-F2 and NE-R2/R primers resulted in a PCR band of 100 bp (Fig. 1). These results indicate that the *Mx* gene of the resident wild waterbirds on the CAPD is the same as that found in the chicken and wild duck.

A number of studies to determine the presence of the *Mx* gene in varieties of chickens and ducks were conducted using the primer identified [19]. The results of these studies showed that the Mx gene is present in a 100 bp fragment [7,14,18,20-21].

The role of the *Mx* gene in chickens began to be analyzed at the time of the AI subtype H5N1 global epidemic. The presence of a chicken population that survived the AI virus infection fostered analysis of the *Mx* gene [15,17]. Studies carried out using the PCR-RFLP method revealed that polymorphism of the *Mx* gene in chickens is caused by exposure to or infection with the AI virus [2,18-22,25]. In addition to chickens, polymorphism of the *Mx* gene is also found in pigs. Using the same method (PCR-RFLP) it was revealed that polymorphism in swine resulted from *Mx* gene exposure to the VSV [9] and influenza virus [26].

Polymorphism in chickens is indicated by the presence of three different variations identified via PCR-RFLP using multiple enzyme cuts. The variations in the cuts from the chickens that were made using the RsaI enzymes were: i) resistant (allele A; genotype AA), ii) resistant/sensitive (allele R; genotype AG), and iii) sensitive (allele G; genotype GG). Using the RsaI cutting enzyme, studies have shown that resistance results in a band cut in a 100 bp fragment, resistant/sensitive results in two pieces in a 100 bp and a 73 bp fragment, respectively, and sensitive produces one piece in a 73 bp fragment [14,19-20].

Such variations indicate differences in the antiviral response of the body to the invasion of the AI virus subtype H5N1. Variations in the antiviral activity of the *Mx* proteins that make up the *Mx* gene are determined by the amino acid asparagine at position 631. They show very high antiviral activity and form the resistance A allele (genotype AA) and the amino acid serine, which is associated with low antiviral activity and establishes the sensitive G allele (genotype GG) [13,27].

In this study, the PCR-RFLP analysis of the DNA from muscovy, duck, and wild water birds located in the CAPD using the RsaI enzyme showed that all the cuttings are contained in a 73 bp fragment (Fig. 2). These results show that no polymorphism has occurred in the resident wild waterbirds and the domestic waterfowl (muscovy and ducks) at CAPD. In the chicken, the cut fragment at 73 bp with the GG genotype showed low antiviral activity because it possesses an *Mx*-sensitive gene (Mx^-) and is susceptible to the AI virus subtype H5N1. The same was not found in the duck, muscovy, and wild waterfowl. Despite having only one strand similar to the Mx^- gene in chickens, there was no indication of dead or sick birds at CAPD due to exposure to the virus.

Different DNA samples from chickens at CAPD showed the strand cut at two locations, namely at 100 bp and 73 bp or at genotype AG (Fig. 2). Variations in the antiviral response of chickens to the influenza virus can be seen in the polymorphism of the *Mx* gene caused by exposure to or infection with the AI virus subtype H5N1. The presence of the AG genotype or the R allele in chickens at CAPD with the resistant/sensitive *Mx* gene confers resistance to the AI virus subtype H5N1. Chickens with these genotypes will generally survive an AI virus subtype H5N1 attack because they have a natural defense mechanism to deal with the AI virus [18].

The results from research by Li & Lu prove that the *Mx* protein from all types of wild ducks only contains serine (AGC/AGU) at amino acid codon 631 [13].Generally,

chickens with the *Mx*- gene would be vulnerable to an AI virus attack [19], and exposure to the AI virus subtype H5N1would result in significant mortality. This is not the case with ducks and wild waterbirds. The *Mx* protein from duck indicated no difference or increase in antiviral activity following exposure to the virus, and it does not show the three variations that are apparent in chickens. In relation to viral attacks, the *Mx* protein is thought not to be active in ducks and not to play a role in inhibiting viral activity because it does not launch an antiviral response when the virus invades the body [28].

However, some studies have shown that the *Mx* protein in ducks and wild birds has a role similar to that of the *Mx* protein in chickens where it regulates the innate immune response. The *Mx* protein in ducks also plays an active role in combating the influenza virus, although it involves a different mechanism than that used by the *Mx* protein in chickens. Although it involves different antiviral activities, the innate immune mechanisms of the *Mx* gene in ducks and wild waterbirds play an important role in limiting infection from the AI subtype H5N1 virus [2,5-6,16,25].

Results of research conducted by Dillon & Rustandler on the diversity of the *Mx* gene in five duck species in Alaska showed a relatively high diversity of nucleotides in *Mx* genes originating from *Anas creccacarolinensis, A. americana, A. platyrhynchos, A. Acuta,* and *A. Clypeata*. Overall, 16 different unique protein sequences were identified in the *Mx* gene from the five duck species. The maintenance of different levels of unique protein sequences and nucleotide diversity at the *Mx* locus is thought to affect the immune response of specific species, with the duck having greater natural immunity than the chicken [16]. As already shown, the antiviral activity of *Mx* proteins varies between organisms [8,9].

Many factors, including the characteristics of the host and the virus, influence mechanisms of resistance or susceptibility to a disease. Despite the number of studies of genes that affect innate immune responses and the role they play in fighting AI viral infections, there are still other factors that may influence the organism's capacity to resist exposure and viral infections [1,15,17,25].

4. Conclusions

The *Mx* gene was present in resident wild waterbirds and waterfowl in a 100 bp fragment, but there was no polymorphism caused by exposure to the AI virus subtype H5N1. The *Mx* gene in resident wild waterbirds regulates the resistance to exposure to the AI virus.

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