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Research Article

Survey on the Presence of the *Mx* and *MHC* Resistance Alleles to Avian Influenza Virus Infection Compared with its Outbreaks Among Chicken Breeds in Egypt

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Abstract

A parallel survey for the incidence of AI in chicken breeds in Egypt including 12 breeds was performed, together with the screening for the presence of their *Mx* and *MHC* alleles. Four years' worth of records of occurrences AI types extending from 2014 to 2017 were used. Also genotyping for the 310 birds under the study using PCR, RFLPs and DNA sequencing was performed. The Baldi (native) chickens were the most affected with all types of AI, especially H5 and H9. In addition, AI was observed in the commercial broilers rather than the layers. Regarding the *Mx* genotypes, although the only detected genotype was the resistance one (Mx/A+) among the Baldi, Cobb and Ross, they were the most affected with AI. Regarding the *MHC* haplotypes, neither the B21 (resistance allele to highly pathogenic AI [HPAI]) nor B13 (susceptible allele) was detected among the studied birds. The homozygous genotypes B4/B4 and B11/B11 were the most common, and new *MHC* alleles were recorded. We recommend recording the names of chicken breeds in AI outbreaks, which will facilitate identification of susceptible breeds in AI outbreaks rather than experimental infection. Further study should be carried out on the most affected breeds with AI to explore their role in the disease epidemiology and whether it is recommended to limit their use during winter, when AI outbreaks occur.

Keywords: *Chickens, HPAI, MHC gene, Mx gene*

Kanatlı İnfluenza Virus Enfeksiyonunda *Mx* ve *MHC* Dayanıklı Allellerinin Mevcudiyeti ve Mısır'daki Tavuk Cinslerindeki Salgınlarla Karşılaştırılması

Öz

Bu çalışmada, Mısır'da toplam 12 tavuk cinsinde Kanatlı İnfluenza insidansı ile birlikte *Mx* ve *MHC* allellerinin varlığı araştırıldı. Çalışmada, 2014 ile 2017 yılları arasındaki dört yıllık periyotta meydana gelen Kanatlı İnfluenza vakaları kullanıldı. PCR, RFLP ve DNA sekans analizi kullanılarak 310 tavuğun genotiplendirilmesi gerçekleştirildi. Baldi (yerel) tavukları H5 ve H9 başta olmak üzere tüm kanatlı influenza tiplerinden en çok etkilenen idi. Ayrıca, Kanatlı influenzası ticari broiler tavuklarda yumurtacılardan daha ziyadesi ile gözlemlendi. *Mx* genotipi ile ilgili olarak, Baldi, Cobb ve Ross cinsleri arasında dirençli olan tek genotip (Mx/A+) olmakla beraber, bu cinsler kanatlı influenzasından en çok etkilenenlerdi. *MHC* haplotipi ile ilgili olarak, ne B21 (oldukça patojenik Kanatlı İnfluenza dayanıklı allel) ne de B13 (duyarlı allel) çalışmadaki kanatlılar arasında tespit edildi. Homozigot genotipler B4/B4 ve B11/B11 en yaygın olup yeni MHC allelleri de belirlendi. Kanatlı İnfluenza salgınlarında tavuk cinslerinin adlarının kayıt edilmesi deneysel enfeksiyonlardan daha ziyadesi ile salgınlarda duyarlı cinslerin belirlenmesinde faydalı olacaktır. Kanatlı influenzasına en duyarlı cinslerin tespitine yönelik ileri çalışmalara hastalığın epidemiyolosindeki rolünün açığa çıkarılması amacıyla ihtiyaç duyulmakta olup bu cinslerin hastalık salgınlarının meydana geldiği kış aylarında kullanımının kısıtlanması tavsiye edilebilir.

Anahtar sözcükler: *Tavuk, HPAI, MHC geni, Mx geni*

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INTRODUCTION

Avian influenza virus (AIV) is a highly contagious viral infection that causes high mortality in chickens. AI A subtypes H5N1 and H9N2 are endemic in Egypt, and the outbreaks of H5N1 have been associated with human infections, especially in 2014-2015. Several genetic markers have been associated with birds' survival from AIV outbreaks in endemic regions [1,2]. The chicken major histocompatibility complex *(MHC)* is known to have a strong association to disease resistance and susceptibility to numerous pathogens [2,3], and this association has been identified in some laboratory and commercials chicken flocks. A set of 27 *MHC* haplotypes were identified in the white Leghorn breed, but little *MHC* information is known for other chicken breeds [4]. The homozygous and heterozygous B21 allele *(MHC* haplotype) is associated with a 100% survival rate from AI outbreaks in Thai indigenous chickens, whereas B13 has a 100% mortality rate [5]. Chazara et al.[6] studied the LEI0258 marker which is located in the B region of the chicken *MHC* and is becoming the reference marker for *MHC* genotyping in chickens.

Mx proteins confer resistance to different virus families; Lee and Vidal [3]; Watanabe [7] demonstrated the *Mx* antiviral activity in response to influenza viruses. In addition, Ko et al.[8] reported one non-synonymous substitution (S631N) in the chicken *Mx* associated with resistance to AIV infection. In addition, Sartika et al.[9] used the mismatching PCR-RFLP method in the *Mx* gene to determine whether chickens carry positive or negative virus activity. Furthermore, the genetic distribution and polymorphism analysis of the *Mx* gene locus as a genetic marker for AI resistance in many indigenous chicken breeds has been documented in several studies [9,10]. The aim of this study was to screen for the presence of different *Mx1* and *MHC1* alleles in chicken breeds in Egypt, especially those reported to be associated with the resistance to AI, as well as the incidence of different types of AIV outbreak in chicken breeds present in Egypt, including 2 native and 10 commercial broiler and layer breeds.

MATERIAL and METHODS

Data Collection

Four years' worth of records of AI A occurrences were obtained from the reference laboratory for veterinary quality control on poultry production, Animal Health Research Institute, Damanhur branch. The records comprise types of AI outbreaks, including both the highly pathogenic AI (HPAI; H5 and H9) and low pathogenic AI (LPAI; H7 and common influenza) from 2014 to 2017 (January-May for each year). The data included in the record represent the number of affected poultry farms from five different Egyptian governorates, the record also includes the breed of the bird.

The virus diagnosis at the reference laboratory depends on the extraction of RNA from pooled homogenate obtained from each flock using and qPCR protocol for virus diagnosis.

Birds Used and DNA Extraction

Blood samples were collected from 310 birds representing 12 chicken breeds from five different Egyptian governorates. The used birds were either native breeds, such as Baldi and Fayoumi (layers) or foreign commercial broilers (Ross, Cobb, Sasso, Hubbard and Arbor) or layers (Hy-line, H&N, ISA, Tetra and Lohman), the number of birds from each breed and their locations are listed in *Table 1*. The blood samples were collected from the brachial vein in the wing area in sterile tubes containing EDTA, transferred to the lab and kept frozen at -20°C. All the birds were handled in accordance with the recommendations of the Committee on the Ethics of Animal Experiments of Kafrelsheikh University, Egypt. Genomic DNA was extracted from whole blood using thermo scientific kits according to the manufacturer's protocol. The purity of genomic DNA was assessed on 2% agarose.

Genotyping of Mx Gene

PCR-RFLP mismatched primers were used for genotyping of the G/A SNP as described by Ommeh et al.[11]. The used primer, annealing temperature, and PCR product size are listed in *Table 2*. *Rsa I* (Thermo Scientific) was employed to cut at the polymorphic (G/A) site, yielding one visible fragment of either 101 bp for allele A or at 73 bp for allele G. PCR conditions for conventional PCR was done using an initial denaturation at 95°C for 5 min, followed by 35 cycles at 95°C for 1 min, annealing temperature *Table 2* for 1 min and 72°C for 1 min, and completed by a final extension at 72°C for 5 min. PCR products were analyzed by electrophoresis through 2% agarose. *Rsa I*(1 U/µg) reaction mixture was carried out according to the manufacturer's protocol, and the fragments were separated on 4% agarose.

Table 1. *Breeds used in this study, their location and number*

No	Chicken Breed	Site of Collection	Number
1	Baldi	Aldelngat, Elbeheira Fowa, Kafr-Elsheik	35 30
2	Sasso	Jankles, Elbeheira	25
3	Cobb	Kafr-Elhenawy, Itay, Elbeheira	30
4	Happered	Alalmia, Alexandria	30
5	Arbor	Almahmodia, Elbeheira	20
6	Ross	Abo-Elmatamir, Elbeheira	20
7	Lohman	Elmansoura- Eldaqalia	20
8	ISA Brown	Gharbia	20
9	H&N	Gharbia	20
10	Tetra	Elmansoura- Eldaqalia	20
11	Fauomy	Damanhour- Elbeheira	20
12	Hayline	Damanhour- Elbheira	20

DNA Sequencing

Mx gene sequences of a representative one bird from each breed under study were performed. The PCR products of *Mx* gene were purified using MEGA quick-spin total fragment DNA purification kit (Intron biotechnology) according to manufacturer instruction. The purified products were sent to LGC Company (Germany). The sequence results were analyzed using Chromas 1.45 (http://www.technelysium.com.au). Sequence comparisons were performed using the BLAST program from National Center of Biotechnology information website http://www.ncbi.nlm.nih.gov. The alignment of obtained sequences done by Clustalw version 1.8.

Genotyping of MHC Gene

The amplified PCR products were used to identify the LEI0258 marker of *MHC +class I αI* and *αIV* (BF) using the genomic DNA [12,13], the PCR reaction mixture and conditions as described in the amplification of *Mx* gene. The primer sequence and the annealing temperature are listed in *Table 2*. The PCR fragments were separated on 4% agarose. Different *MHC* haplotypes were identified as described by Fulton et al.[13].

RESULTS

Based on the four years records, the native breed (Baldi) showed a higher incidence of all types of avian influenza during 2014-2017 *(Fig. 1)*, also, it is the most affected breeds with HPAI (H5 and H9). Cobb broilers were the second affected by AI especially H9, then Sasso breed. Sporadic appearance of the AI in some other breeds as Hubbard during 2014, Ross in 2015 and Arbor during 2016. No AI outbreaks were recorded in Fayoumi. Additionally, no reports of AI among the studied layer breeds were recorded

The genomic DNA of 310 samples was successfully amplified for *Mx* gene with a size product of 100 bp. *Rsa1* was used for identification of resistant and sensitive chicken *Mx* gene based on the fragment size. The homozygous individuals (A/A) with one band at100 bp (resistant *Mx*); heterozygous (A/G) with two bands (100 bp and 73 bp); and a third genotype (G/G), homozygous sensitive *Mx* allelic gene) with one band at 73 bp. Regarding the distribution of these allele among the chicken breeds, AA is the only genotype found in all selected birds of the 10 out of 12 studied breeds, AA frequency in Hy-line is 0.6, AG genotype is 0.3 and GG genotype is 0.1. AA genotype in Fayoumi

Table 2. *Primers used in this study*

Gene	Sequence	Anealing Temp	Size of PCR Product
Mx primer	5′-GAGTACCTTCAGCCTGTTTT-3′ 5′-TGCAAAAACATCTTCAAGTCTCTG-3′	60 C	101 bp
MHC-BFαI	5′-GTGGACGGGGAACTCTTC-3′ 5′TCTGGTTGTAGCGCCG-3′	58 C	Variable
MHC-BFαIV	5′GTGGACGGGGAACTCTTC- 3′ACCGCCGGTCTGGTTGTA-3′	58 C	Variable

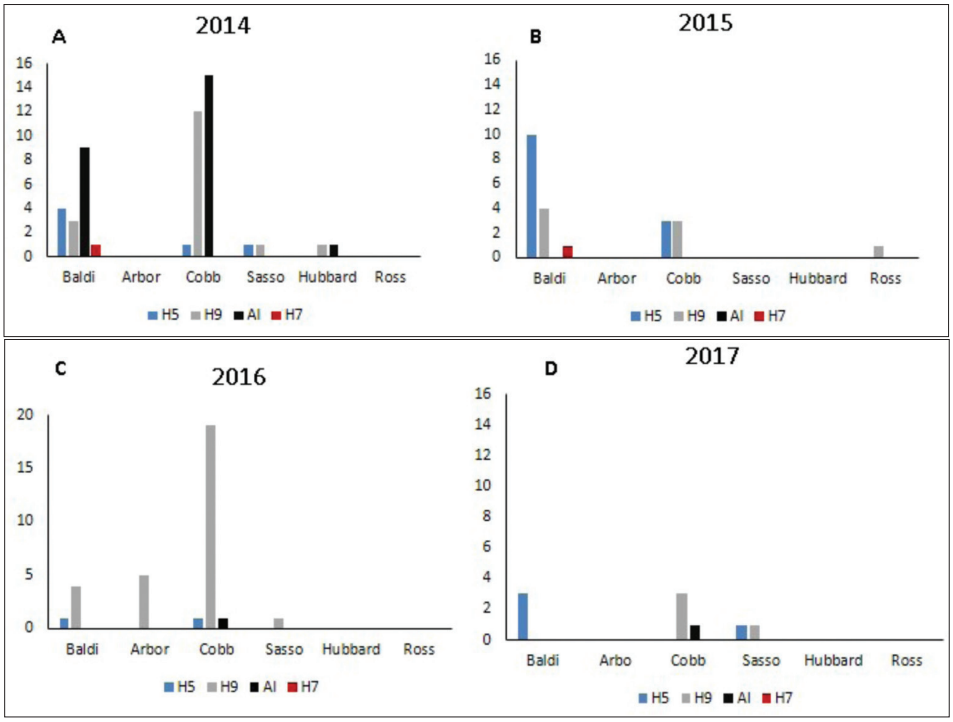

Fig 1. Incidence of different types of Avian influenza A (H5, H9, AI and H7) among chicken breeds in Egypt from 2014-2017

Table 3. *Distribution of resistant and sensitive Mx genotypes in various breeds of chicken based on PCR-RFLP product*

Groups	Breed	Mx Genotype		
		AA/Mx^{++}	AG/Mx^{+-}	GG/Mx^{--}
1	Baldi	65 (1.0)	0	0
2	Fayoumi	10 (0.5)	10 (0.5)	0
3	Hy-Line	12 (0.6)	6 (0.3)	2 (0.1)
4	Cobb	30 (1.0)	0	0
5	Ross	20 (1.0)	0	0
6	Sasso	25 (1.0)	0	0
7	Hubbard	30 (1.0)	0	0
8	Lohman	20 (1.0)	0	0
9	H&N	20 (1.0)	0	0
10	ISA	20 (1.0)	0	0
11	Arbor	20 (1.0)	0	0
12	Tetra	20 (1.0)	0	0

Resistance allele: A/Mx^{+}. Sensitive allele: G/Mx^{-}

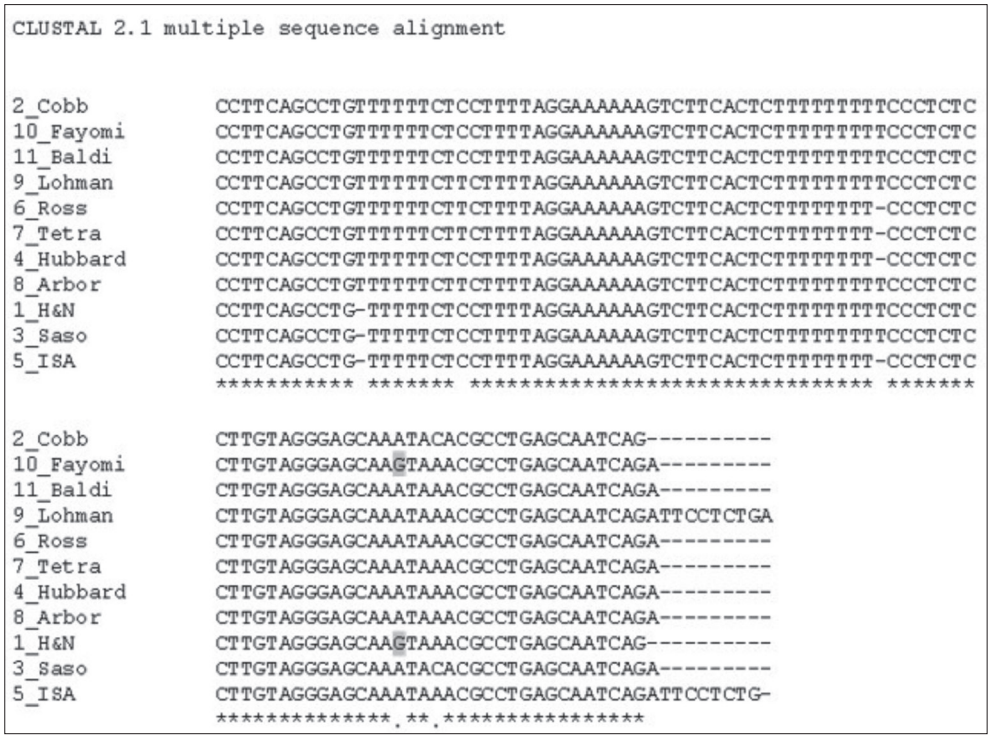

Fig 2. Sequence alignment of *Mx* gene from different chicken present in Egypt

is 50% and AG genotype is 50% *(Table 3)*.

Sequence alignment of Mx gene among the studied chicken breeds indicated the presence of alternative A or G *(Rsa1* recognition site) allele at the nucleotide number 73 of the amplified sequences. The A allele is present at Baldi, Sasso, Ross, H&N, Arbor, Hubbard, ISA, Cobb, tetra, and Lohman. G allele is present at Fayoumi and Hy-line *(Fig. 2)*.

Following the amplification and identification of the LEI0258 alleles of various B haplotypes of *MHC* in different chicken breeds, they are listed in *Table 4*. For *MHC-BFaI* haplotype, three genotypes were repeated among the studied chicken breeds; B 4 (182 bp) was the most common allele present either in the homozygous (B4/B4) in Fayoumi, Sasso, Hubbard, and Arbor, or heterozygous with other new alleles B-N1 750 bp or BN2 800 bp. among the other breeds. Regarding the *MHC-BfaIV haplotypes,* homozygous genotype (B11/B11) was the only genotype detected among Fayoumi, Hy-line, Sasso, Hubbard, Tetra, and Cobb. Heterozygous genotype, B11/B12, B11/B24, B11/B12.3 were detected among Baldi, H &N, Ross and Arbor chickens *(Table 4)*. Neither B21 allele (resistance allele to HPAI) nor B13 alleles (susceptible allele) were detected among the studied breeds

Table 4. *Distribution of LEI0258 alleles of defined MHC (MHC-BFaI and BfaIV) haplotypes in different chicken breeds under study*

Breed		*MHC-BFaI* Haplotype			*MHC-BFaIV* Haplotype			
		B4/B4 182/182	B4/B-N1 182/750	B4/B-N2 182/800	B11/B11 193/193	B11/B24 193/309	B11/B12 193/478	B11/B12.3 193/513
1	Baldi	26	26	13	33	32	0	0
2	Fayomi	20	0	0	12	0	0	0
3	H-Line	16	4	0	18	2	0	0
4	Cobb	20	10	0	10	20	0	0
5	Ross	0	20	0	5	0	20	0
6	Sasso	25	0	0	20	1	5	0
7	Hubbard	30	0	0	25	0	0	5
8	Lohman	0	20	0	0	20	0	0
9	H & N	7	13	0	13	7	0	0
10	ISA	0	20	0	20	0	0	0
11	Arbo	20	0	0	10	10	0	0
12	Tetra	0	15	5	20	0	0	0

DISCUSSION

Although, many reports suggests that native chicken breeds are more resistant to AIV infection, however, no data available supporting this suggestion [14]. Our data indicate that the Baldi chickens (native Egyptian breed) was the most affected with all types of AIV especially H5 and H9 along the records extends from 2014-2017. Also, Sims et al.[15]; Ellis et al.[16] demonstrated that 'local' Chinese breeds of chicken, exposed to an infectious dose of HPAI H5N1 die such as observed in some naturally-infected commercial flocks. On the other hand, Fayoumi another important native layer breed, with an absence record of avian influenza viruses. Additionally, a high incidence of Avian influenza was also recorded in only the commercial broilers as Cobb chickens which is the second affected by avian influenza, then Sasso, Hubbard, and Arbor. No reports of AI among the studied layer breeds were recorded. Reversely, Bertran et al.[17] suggested that broiler breed is less susceptible to the H5N2 virus than the layer breed.

Regarding *Mx* genotypes, variation in *Mx* allele frequency among the two studied native breeds (Baldi and Fayoumi) was observed, and the resistance genotype is the only demonstrated one among 10 out of 12 breeds, however, in the Hy-line and Fayoumi, both have resistance and sensitive alleles. Fadhil and Mercan [18] demonstrated that the resistant *Mx* gene allele (A/Mx+) frequency was 98% and the sensitive allele frequency (G/Mx-) was 2%, in Gerze Turkish chicken breed while in pure line chicken breed, *Mx* sensitive allele frequency was 48% and the resistant allele frequency was 52%. Hassanane et al.[19] demonstrated that the average allele frequency of the resistant A allele in some Egyptian chicken breeds was higher than the sensitive G allele, and the resistance allele A is the only allele present in Egyptian Baldi chickens which is in agreement with our results.

Many reports have investigated the two allele A/G substitution polymorphism coding amino acid position 631 of *Mx* gene and its role in resistance to AI [7,20]. However, Sironi et al.[21] indicated that the *Mx* genotype did not affect the clinical status or the time course of infection after viral experimental infection of five chicken lines with one of HPAI virus. Furthermore, Sironi et al.[22] in a genomic study to the response of chicken to HPAI virus, concluded that neither the genotype at the *Mx* gene or *MHC-B* locus, involved in variations to response to AIV infection. However, many reports have been documented the role of chicken's major histocompatibility complex (*MHC*) haplotype on the resistance or susceptibility of HPAI [5]. Hunt et al.[23] challenged a series of *MHC* congenic white leghorn chicken lines with a low dose of (H5N1) virus, they demonstrated that none of the lines were completely resistant to the lethal effects of the challenge.

Regarding the *MHC* haplotypes in our study, neither B21 allele (resistance allele to HPAI) nor B13 alleles (susceptible allele) indicated by Boonyanuwat et al.[5] were detected among the birds included in this study. Additionally, few number of alleles are detected and the homozygous genotypes B4/B4, B11/B11 are the most common especially among Fayoumi, Sasso, and Hubbard. Heterozygous genotypes were detected among the other breeds with newly detected haplotypes. Shavakand [24] detected less variation in microsatellite markers (LEI0258), situated within the *MHC* region, in the industrial chicken populations compared to non-industrial populations.

Comparison between the data of AI incidence with the genotypes of chickens under the study, Baldi breeds, Cobb and Ross were the most affected, although they only have the resistance genotype (Mx/A+). On the other hand, Fayoumi chickens another native chicken breed with no reports of avian influenza have both resistance and

susceptible genotypes with 50% of each. Matsuu et al.[25], observed absence of a significant association between the *Mx* and *MHC* class I genes polymorphisms in these loci and resistance to HPAIV.

In a survey about HPAI introduction into Europe by EFSA supporting publication [26], at the period 2005-2015, the species affected by HPAI outbreaks were frequently reported as ducks, mixed ducks and geese, and undetermined species of backyard flocks; however, no data about the chicken breeds are included. Taking into consideration the consumer demand and the related economic losses of Avian Influenza outbreak [27], we suggest that the determination of the most susceptible chicken breed, would have great impact in controlling the disease and subsequently the economic losses.

In a conclusion, we recommend recording the names of chicken breeds in AI outbreaks, which will facilitate identification of susceptible and resistance breeds in AIV outbreaks rather than experimental infection. Further study should be carried out on the most affected breeds with AI to explore their role in the disease epidemiology and whether it is recommended to limit their use during winter, when AI outbreaks occur.

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