Identification of Mx gene nucleotide dimorphism (G/A) as genetic marker for antiviral activity in Egyptian chickens

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1. Introduction

Mx proteins are Damin-like guanosine triphosphate metabolizing enzymes (GTPases). They are the products of interferon-stimulated Mx genes which exist in almost all vertebrates ranging from fish to humans [1–3]. Mx proteins are known to be involved in inhibiting the multiplication of several RNA viruses, including Orthomyxoviridae, Paramyxoviridae, Rhabdoviridae, Bunyaviridae and Togaviridae as well as some DNA viruses, including Hepadnaviridae [4–10]. Mx proteins are members of the large GTPases family. These GTPases share an N-terminal GTPase domain, a middle domain (MD), and a C-terminal GTPase effector domain (GED). The MD and GED are very important for the conformation and activity of the Mx proteins. The MD is important for oligomerization and virus target recognition [11]. On the other hand, the GED functions as an intramolecular GTP-activating domain: the C-terminal leucine zipper motif (65–70 amino acids) in the GED folds back to join the N-terminal GTP-binding domain, forming the enzymatically active center of Mx proteins [12]. Some vertebrates have up to three Mx gene copies in the same organism. For example, mice and humans carry more than one Mx gene [13].

Chickens have a single Mx gene (Mx1) that is induced by type I interferon [14]. The Mx gene is located on chicken chromosome 1 in approximately 20767 bp fragment and consists of 14 exons. The Mx mRNA or cDNA molecule length is 2545 bp with a 2115 bp coding region that codes for 705 amino acids protein [15,16]. The chicken Mx protein has been reported to confer antiviral activity against the influenza viruses from the Orthomyxoviridae family and the recombinant vesicular stomatitis virus (VSV) from family Rhabdoviridae [17–21]. It has been also reported that Mx protein exhibited antiviral activity against the Newcastle Disease virus...
(NDV) from the Paramyxoviridae family [22,23]. A non-synonymous dimorphism G/A in exon 14 of the chicken Mx gene (this position corresponds to nucleotide number 2032 in Mx cDNA reference sequence accession number: Z23168) results in the presence of serine (Ser) or asparagine (Asn) at amino acid (aa) 631, located in the C-terminal GTPase effector domain of the Mx protein. The substitution of Ser with Asn and vice versa alters the Mx protein’s antiviral activity [19].

Several in vitro studies have indicated that the presence of Asn at aa 631 (Asn631 allele), which results from homozygous AA genotype, had higher antiviral activity than the presence of Ser631 allele, from GG individuals, against VSV and NDV in chickens [19,20,22,24]. Sasaki et al. [24] also found that replacing Ser with Asn at 631 aa altered intracellular localization of the Mx protein. Moreover, Yin et al. [22] reported that the AA individuals from Beijing-You and White Leghorn chicken breeds had higher Mx expression levels than GG individuals. The higher antiviral activity of the AA genotype against the ND virus was confirmed in vivo study by Pagala et al. [23]. They confirmed that AA and AG genotypes were resistant against NDV and showed better production than GG genotype in Tolaki chickens.

Several studies have also presented conflicting results regarding whether or not the substitution of Ser with Asn in the Mx protein at the aa 631 (Ser631) is associated with resistance to avian influenza virus infection in chickens [25]. In an in vitro study by Ko et al. [19] demonstrated that Ser631 substitution in the Mx protein confers more resistance to the highly pathogenic avian influenza (HPAI) H5N1 subtype in chickens. In an in vivo study, Ewald et al. [26] reported that chickens homozygous for Asn631 allele were significantly more resistant to disease based on early mortality, morbidity, or virus shedding than Ser631 homozygotes when infected with HPAI H5N2 subtype. Wang et al. [27] also reported that chickens with AA genotype had high gene expression and a non-significant tendency for lower virus titer, when infected with influenza virus H5N3 subtype, than GG individuals. Two other groups have failed to support the anti-influenza activity of chicken Mx Asn631 against influenza virus subtypes H1N1, H4N6, H5N1, H5N3 and H7N1 in vivo and in vitro systems [28,29].

Despite the previously mentioned conflicting results regarding the anti-influenza activity of chicken Mx Asn631, the latter could be important for chicken response to other viruses. Therefore, it is possible to breed infectious disease-resistant chickens that carry the A allele at the above-mentioned nucleotide position, which could help to prevent the spread of viral infections.

Egyptian chickens are grouped into around 15 breeds and strains. Egyptian chickens have a small body and a dual purpose for meat and egg production. They have some useful genetic attributes such as adaptability to local environment, resistance to some diseases, possessing a good nicking ability and lower clutch [30]. Genetic improvement programs for poultry breeding in Egypt will be of a great economic importance. Therefore the aim of the present study was to identify the Egyptian chickens that carry the Mx gene resistant allele with the high frequencies using the PCR-RFLP genotyping and sequencing techniques. The obtained data could help in improving poultry breeding in Egypt by producing infectious disease-resistant chickens.

2. Materials and methods

2.1. Chicken samples

The study was performed on 246 Egyptian chickens, representing 2 breeds Dandarawi and Fayoumi in addition to 7 strains: El-Salam, Golden Montazah, Dokki-4, White egg commercial, Red egg: commercial, Gemmizah and Baladi.

2.2. Blood sampling

The blood samples were collected from brachial vein in the chicken wing area in sterile tubes containing 0.5 ml EDTA, transferred to the lab and kept frozen until DNA extraction. Details of the collected blood samples are presented in Table 1.

2.3. DNA isolation and quantification

DNA was extracted from whole blood using commercial Kit (GeneJET Whole Blood Genomic DNA Purification Mini Kit, K0781) according to the manufacture instructions. DNA concentration was measured using UV spectrophotometer (Shimadzu UV 2401) at 260 nm wavelength.

2.4. PCR amplification and visualization

PCR was performed using the primer set designed by Sironi et al. [31] for the amplification of a DNA fragment (299 bp or 301 bp) from the Mx gene that contains the target dimorphic nucleotide position G/A. The primers sequences were: forward (F) 5’-GCACGTCAACCTTATTAGA-3’ and reverse (R) 5’-GTATTGAGCCTTTTCTTGA-3’. The F primer anneals to the last intron of the Mx gene, and the R primer anneals to the last exon of the gene. PCR was carried out in a total volume of 25 μL, with 50 ng genomic DNA, 10 pmol of each primer, 2.5 μL 10X buffer, 1.5 mM MgCl2, 0.2 mM of each dNTP, and 1 U from Dream Tag (Thermo Scientific). The reaction was accomplished in TM Thermal Cycler (MJ Research PTC-100 thermocycler, USA). The thermal cycling was as follows: initial denaturation step at 95 °C for 4 min followed by 35 cycles.
of 94 °C for 1 min, annealing at 54 °C for 1 min, and extension at 72 °C for 1 min, followed by a final extension step at 72 °C for 10 min. PCR successful products were identified on 2% agarose gel in 1X TBE buffer and visualization after staining with ethidium bromide (EtBr). All polymerase chain reactions were conducted in duplicates.

2.5. Genotyping

The PCR products were digested with restriction enzyme Hpy81 (Fermentas). The digestion reaction mixture was prepared using 10 μl of PCR product, 10 U restriction enzyme, dH2O and Tango buffer according to the manufacturer instructions, the mixture was incubated at 37 °C for 15 min. Then the enzyme was deactivated by incubating at 65 °C for 10 min. In case of the G allele presence, the enzyme cuts the sequence 5'-GTN|NAC-3', 2 bp downstream of the target Mx dimorphism, whereas the fragment containing the A allele is not cleaved. Digestion products were subjected to electrophoresis on 3% agarose gel in 1X TBE buffer and visualized after staining with ethidium bromide. Genotype and allele frequencies for the two alleles A and G were calculated in all the Egyptian chickens. Genotype frequencies were calculated according to Brooker et al. [32]. The allele frequencies were calculated from the genotype frequencies according to Gillespie [33].

2.6. Sequencing

PCR products were purified using the Exo SAP-IT PCR Purification Kit (Applied Biosystems) following the manufacturer’s recommended protocol. Sequencing reactions were performed using Big Dye TM terminator Cycle Sequencing Kit (Applied Biosystems). Sequences were determined using ABI3700 and 3730 automated DNA sequencers (Applied Biosystems).

2.7. Protein translation and sequence analysis

DNA sequences were translated to proteins by using the ExBASy-Translate tool [http://web.expasy.org/translate]. Nucleotide sequence alignments were performed using CLUSTALW program [http://www.genome.jp/tools/clustalw/].

3. Results

3.1. The Mx gene amplified fragment

The size of the Mx gene PCR amplified fragment that containing the target dimorphic position G/A was 299 bp or 301 bp (Fig. 1). The exact size of each amplified fragment was determined from sequencing results.

3.2. Genotype and allele frequencies

Cutting the PCR products with the restriction enzyme Hpy81 produced two bands (at approximately 200 bp and 100 bp) in the homozygous samples that possess allele G only (GG genotype). The uncut PCR products were at approximately 300 bp representing the homozygous samples with allele A only (AA genotype). The three bands at approximately 100 bp, 200 bp, 300 bp represented heterozygous samples (AG genotype) (Fig. 2).

The genotype and allele frequencies of the A and G alleles in the different studied chickens are presented in Table 2. The three genotypes AA, AG, GG of the Mx gene were represented in all the tested Egyptian chicken breeds and strains except the Balabi strain which showed only one genotype AA, with a genotype frequency value of 1.000. The highest frequencies of GG and AG genotypes were 0.473 in the El-Salam strain and 0.842 in the Red egg commercial strains, respectively. The average AA, AG and GG genotype frequencies in all the tested chickens breeds and strains were 0.44, 0.45 and 0.11 respectively. The obtained results indicated that the A allele was presented in all the tested chicken breeds and strains. The calculated allele A frequency values ranged from 0.417 in El-Salam strain to 1.000 in Baladi strain. The G allele was also detected in all the tested chickens, except Baladi strains, with allele frequency values that ranged from 0.197 in Fayoumi strain to 0.583 in El-Salam strain. Results also indicated that the average A allele frequency in all the tested chicken breeds and strains (0.67) was higher than the average G allele frequency (0.33).

3.3. Sequence pattern

Sequences of the amplified Mx gene fragment which obtained from the different Egyptian chicken breeds and strains samples were grouped in five sequence patterns. The five sequence patterns were submitted to the International genbank database under the
accession numbers: KY584063, KY584064, KY584065, KY584066 and KY584067. Alignment result of the five detected sequence patterns is presented in Fig. 3.

The results indicated full identity between the data collected by PCR-RFLP and sequencing techniques for the presence of A or G alleles at the target dimorphic position in \(Mx\) gene exon 14. This position is at the nucleotide No. 99 in the amplified \(Mx\) gene fragment and corresponds to the nucleotide number 2032 in the previously mentioned \(Mx\) cDNA reference sequence. Representatives of sequencing data from the AG, GG and AA genotypes at the nucleotide position No. 99 are shown in Fig. 4.

In addition to the previous G/A dimorphism, four variable sites were also detected within the \(Mx\) gene amplified fragment from different samples (Fig. 3). Three of which were in the intronic region: one C/T dimorphism is at nucleotide number fourteen and two successive insertion/deletion are after the nucleotide number 71, of the amplified fragment. The fourth one was another G/A dimorphism in the \(Mx\) exon 14 at the nucleotide position No. 226 of the amplified fragment. This position corresponded to the nucleotide number 2159 in the \(Mx\) cDNA reference sequence.

Protein translation of the coding exonic region in the different detected \(Mx\) gene sequence patterns indicated that the dimorphism G/A at the position that corresponding to nucleotide number 2032 in \(Mx\) cDNA is the only non-synonomous dimorphism. It results in a change at amino acid 631 of the \(Mx\) protein from serine to asparagine and vice versa. The other detected dimorphism G/A in the same amplified coding region was found to be synonomous.

### 4. Discussion

Due to the severe threats of infectious spread to large numbers of livestock, animal breeding focused on innate immune-associated genes such as \(Mx\) that may play an important role in reducing the incidence of infection. The \(Mx\) pathway is one of the most powerful pathways. The \(Mx\) protein has a direct antiviral activity that inhibits a wide range of viruses by blocking an early stage of the viral replication cycle [22]. In chickens, the \(Mx\) gene protein product has been reported to confer antiviral activity against the influenza viruses, the recombinant vesicular stomatitis virus (VSV) and Newcastle Disease virus (NDV) [17–23].

Sequence analysis of \(Mx\) cDNA from Japanese and Egyptian chickens revealed that the non-synonomous dimorphism (G/A) in \(Mx\) gene exon 14 leads to the change at aa 631 of the chicken \(Mx\) protein. This aa change was found to affect \(Mx\) protein antiviral activity [19]. The amino acid 631 is found in the C-terminal GTPase effector domain of the \(Mx\) protein (GED) that is very important for the activity of the protein. The change at the aa 631 is from

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**Table 2**

| Chickens            | No. | Genotype frequency | Allele frequency |
|---------------------|-----|--------------------|------------------|
|                     |     | AA    | AG   | GG  |     | A      | G      |
| Dandarawi breed     |     | 0.750 | 0.250| 0.000 |     | 0.875  | 0.125  |
| Male                | 8    |        |      |      | 0.567 | 0.333  | 0.100  |
| Female              | 30   | 0.605 | 0.316| 0.079 |     | 0.763  | 0.237  |
| Total               | 38   | 0.500 | 0.400| 0.100 |     | 0.700  | 0.300  |
| Fayoumi breed       |     | 0.714 | 0.250| 0.036 |     | 0.840  | 0.160  |
| Male                | 10   |        |      |      | 0.714 | 0.249  | 0.037  |
| Female              | 28   | 0.658 | 0.289| 0.053 |     | 0.803  | 0.197  |
| Total               | 38   | 0.500 | 0.400| 0.100 |     | 0.700  | 0.300  |
| El-Salam strain     |     | 0.333 | 0.333| 0.333 |     | 0.590  | 0.410  |
| Male                | 6    | 0.333 | 0.333| 0.333 |     | 0.590  | 0.410  |
| Female              | 30   | 0.300 | 0.200| 0.500 |     | 0.400  | 0.600  |
| Total               | 36   | 0.305 | 0.222| 0.473 |     | 0.417  | 0.583  |
| Golden Montazah strain |     | 0.429 | 0.429| 0.143 |     | 0.643  | 0.357  |
| Male                | 14   | 0.429 | 0.429| 0.143 |     | 0.643  | 0.357  |
| Female              | 18   | 0.222 | 0.611| 0.167 |     | 0.528  | 0.472  |
| Total               | 32   | 0.313 | 0.531| 0.156 |     | 0.578  | 0.422  |
| Dokki-4 strain      |     | 0.250 | 0.750| 0.000 |     | 0.625  | 0.375  |
| White egg commercial strain |     | 0.530 | 0.411| 0.059 |     | 0.735  | 0.264  |
| Red egg commercial strain | 19 | 0.105 | 0.842| 0.063 |     | 0.526  | 0.474  |
| Germizah strain     |     | 0.000 | 0.000| 1.000 |     | 0.000  | 1.000  |
| Male                | 1    |        |      |      | 0.000 | 0.000  | 1.000  |
| Female              | 24   | 0.208 | 0.791| 0.041 |     | 0.583  | 0.417  |
| Total               | 25   | 0.200 | 0.791| 0.041 |     | 0.583  | 0.417  |
| Baladi strain       |     | 0.000 | 0.000| 1.000 |     | 0.000  | 1.000  |
| Male                | 11   | 1.000 | 0.000| 0.000 |     | 1.000  | 0.000  |
| Female              | 10   | 1.000 | 0.000| 0.000 |     | 1.000  | 0.000  |
| Total               | 21   | 1.000 | 0.000| 0.000 |     | 1.000  | 0.000  |
Asparagine to serine and vice versa. This aa substitution is a semi-conservative substitution that means the two amino acids share some properties but not all the physiochemical properties. Semi-conservative amino acids substitution may affect protein folding, stability, function, expression as well as protein-protein interactions and sub-cellular localization of the protein molecules [34–37]. Several studies confirmed that the variant having asparagine instead of serine at position 631 is responsible for the raise of the antiviral activity of the Mx protein against VSV, NDV as well as influenza viruses H5N1, H5N2 and H5N3 [19,20,24,22,26,27,23].

Several studies have investigated the frequencies of the two alleles A and G that are responsible for the change at aa 631 in chickens Mx gene using a variety of methods: mismatched polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) [38], electrophoresis of PCR products on polyacrylamide gels [39], and sequencing [40]. In the present study a
PCR-RFLP protocol [31] was applied for the two alleles A and G genotyping. This genotyping protocol simply does not require the creation of mismatches as the protocol of Seyama et al. [38]. The PCR-RFLP results indicated that the three genotypes AA, AG, GG at the target nucleotide position in the Mx gene were represented in all the tested Egyptian chicken breeds and strains except the Baladi strain which showed only one genotype AA. The average AA, AG and GG genotype frequencies in all the tested chickens were 0.44, 0.45 and 0.11 respectively. The present results also indicated that the average allele frequency of the resistant A allele in all the tested birds (0.67) was higher than the sensitive G allele average frequency (0.33). Similar to our results were obtained by Sartika et al. [41]; who reported that indigenous chickens in Indonesia have more frequency of resistant type Mx gene allele. In other studies, it was reported that the GG genotypic frequency was significantly higher than the AA genotypic frequency in Chinese and Bangladeshi chickens [15,16].

The sequencing results indicated complete matching between the results of PCR-RFLP and sequencing techniques in identifying the A or G alleles at the target nucleotide position, that responsible for the change at amino acid 631 in the Mx protein. The other G/A dimorphism at the nucleotide position, that corresponds to the nucleotide number 2159 in the Mx cDNA reference sequence was found to be synonymous. This dimorphism was also identified before by Ko et al. [19] in Japanese and Egyptian chickens Mx cDNA among 25 polymorphic positions.

The interesting finding of the present study is the highest genotype and allele frequencies for the resistant A allele that was detected in the Baladi chicken strain which showed AA genotype only. The Baladi strain originated from hybridization among exotic and Egyptian autochthonous chickens continued along with different times of old trade dispersal and colonization to Egypt [42]. Applying PCR-RFLP technique in the breeding programs to select chickens that carry the Mx resistant allele A from the different Egyptian chicken breeds and strains especially from the Baladi strain will help in raising chickens antiviral activity.

5. Conclusion

In the present study the genotype and allele frequencies of the resistant and the sensitive alleles of the Mx gene were identified in tested Egyptian chicken breeds and strains. The results indicated that the average allele frequency of the resistant A allele is higher than the sensitive G allele average frequency. The highest genotype and allele frequencies for the resistant A allele was detected in the Baladi strain which showed AA genotype only. Selection of chickens that carry the Mx resistant allele as a genetic marker for high antiviral activity in the breeding programs could help in improving poultry breeding in Egypt by producing infectious disease-resistant chickens.

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