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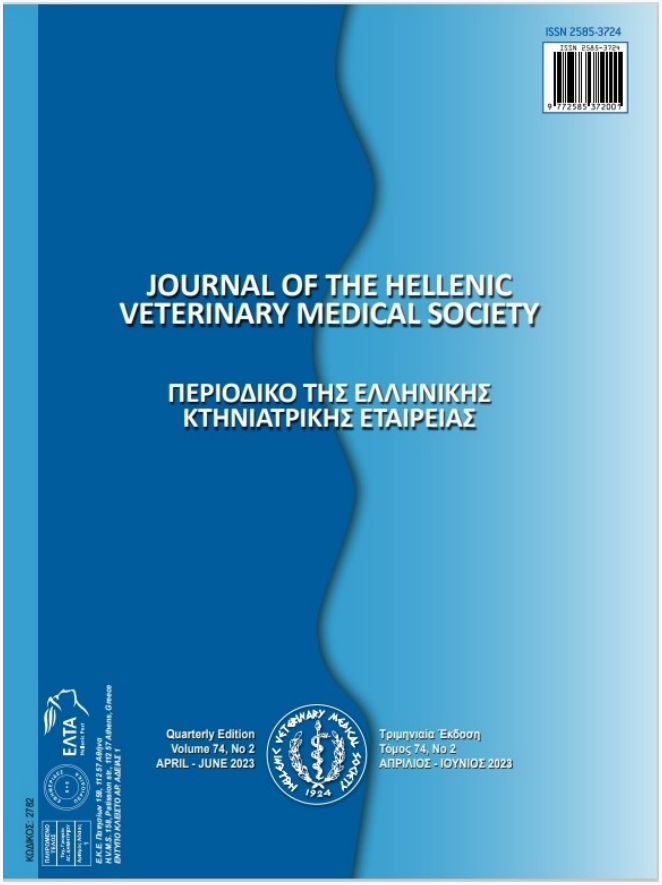


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Research article
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# Identification of single nucleotide variant in *Mx1* gene associated with antibody response to Avian Influenza virus in Aseel chickens

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**ABSTRACT:** Aseel chickens are known for their hardiness, thermotolerance, pugnacity, and robustness. However, they are highly susceptible to Avian Influenza (AI) and Newcastle Disease (ND) viruses which cause huge mortalities. The present study was designed to identify polymorphisms in exon-14 of *Mx1*, and 5'UTR and intron-7 of *ROBO2* gene associated with antibody response to AIV (H9N2) and ND virus respectively in adult Aseel birds. After screening 500 birds for HI-based antibody response against both viruses, two separate sets of 40 birds, one for each virus, were selected on the basis of maximum divergence in their antibody response. The sequence data of exon-14 of *Mx1* gene revealed 3 SNP, and that of *ROBO2* gene showed 2 SNPs in intron-7 and 1 in 5'UTR. The genotypic frequencies of identified variants were tested for goodness-of-fit and only Mx_810 showed significant differences. The results of association analysis revealed a non-synonymous SNP (G>A), Mx1_567, significantly associated with pre- ($P<0.01$) and post-vaccination ($P<0.01$) antibody response against AIV. The LSD results showed that homozygous mutant (AA), and heterozygous (AG) genotypes had significantly greater values of pre- and post-vaccination antibody response compared with the wild-type genotypes. However, no SNP in the *ROBO2* gene was found significantly associated with antibody response.

***Keywords***: SNP; *Mx1* gene; *ROBO2* gene; Antibody response; Avian influenza; Newcastle disease; Aseel chickens

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## INTRODUCTION

Aseel is the most popular and well-renowned chicken breed in Indo-Pak subcontinent which has also got its description in the British, Australian, and American poultry standards (Allonby & Philippe, 2018; Associaton, 2015). Birds of this breed are famous for their stamina, majestic gait, upright posture, pugnacity, and capability of persistent fighting (Rajkumar et al., 2017; Usman et al., 2014). Due to these qualities, the demand of Aseel chickens is very high in South Asian countries, particularly in cock-fighter and fancier communities (Babar et al., 2012; Khan et al., 2016). Moreover, Aseel had also been reported as the ancestors of White Cornish, and Plymouth Rock (Dohner, 2001), the parent of contemporary broilers. These birds are economically important in rural areas due to their vigor, appearance, greater body size, delicious meat, fighting behavior, and adaptability to harsh climatic conditions (Bashir et al., 2014; Rajkumar et al., 2016; Usman et al., 2014). Though Aseel birds are well-known for their resistance to many diseases, but are highly susceptible to Newcastle Disease (ND) (Murree et al., 2016) and Avian Influenza (AI) (Suba et al., 2015).

Avian Influenza (AI) and Newcastle disease (ND) are two lethal viral diseases of poultry, which not only badly affect poultry industries worldwide but also have very negative impacts on the livelihoods of rural farmers, especially in developing countries by causing huge morbidities, and mortalities among the flocks (Liu et al., 2015). The chicken AI viruses are the members of the family Orthomyxoviridae(Zhao & Pu, 2022), and are further categorized into 16 different subtypes on the basis of Haemagglutinin (H) antigen, and 9 different subtypes on the basis of Neuraminidase (N) antigen.

AI had been reported to cause the greatest loss to the poultry industry worldwide (Smith et al., 2015) and this virus can also be transmitted from chicken to human due to its zoonotic properties and hence pose a great threat to human health. The H5N1 and H7N9 subtypes of AI viruses are of major zoonotic importance, however, the most common subtype found in human cases is the H5N1 (Huai, 2008). The rise in human infections by H7N9 virus in China has provoked pandemic concerns over the years. And since 2012, over 1400 cases of human infection with H7N9 have been reported which were due to exposure from poultry only (Uyeki et al., 2017).

However, ND is considered one of the most critical diseases of the birds due to its high contagiousness and mortality rate, pervasive distribution throughout the world and subsequent massive economic losses. In 2011, ND was ranked 4 as the 4$^{th}$ major devastating disease for causing economic losses to poultry industries worldwide (Smith et al., 2015), and number one disease constraint for poultry production in developing countries (Rowland et al., 2018). The occurrence of this disease can be minimized by the vaccination of birds with the less virulent ND viruses, which is the best way to control this disease because there is no remedy for its treatment yet. However, host antibody response plays a vital role in rendering the host resistance to ND. Conventional vaccinations coupled with modern technology seek to protect the birds from fatal pathogens but changes in the pathogenicity of causative agents help them continue their multitude in birds which subsequently lead to massive economic losses (Sivaraman & Kumar, 2013). Therefore, improving genetic resistance of the birds is an alternative and sustainable approach to protect them from the viral diseases.

Moreover, genetic variability exists in chickens for immune response to several antigens like AI and ND viruses (Drobik-Czwarno et al., 2018; Liu et al., 2014; Rowland et al., 2018a). Selective breeding of virus-resistant chickens would be beneficial for both the poultry production and human health, as both of these diseases are zoonotic in nature. Several studies had been conducted to find out the candidate genes and genetic markers for disease resistance traits in chickens and subsequently many candidate genes have been reported for their role in antibody response. Like, *Mx1* gene is a renowned candidate gene for its antiviral activity towards resistance to avian influenza virus (Ko et al., 2002) and *ROBO2* gene is a candidate gene for modifying the antibody response against Newcastle Disease (Luo et al., 2013). The products of Mx genes are part of the innate immunity and help the host to develop resistance against avian influenza (Ko et al., 2002). *ROBO2* gene had also been reported to affect the expression of Rac1 and CDC42 which are vital in T cell development and memory T cell growth (Guo et al., 2011; Smits et al., 2010).

Selection of birds on basis of their resistance to various diseases have ever remained a priority of commercial breeders (Drobik-Czwarno et al., 2018b; Fulton, 2004). However, traditional selection for disease resistance has many challenges including high costs. Therefore, the integration of classical and genomic

approaches for identifying underlying genes and causative variants controlling different traits is highly recommended (Cheng et al., 2013). These variants can be utilized in marker-assisted selection (MAS) programs to overcome the limitations of classical breeding approaches (Goddard & Hayes, 2009; Helal et al., 2021). Accordingly, more attention should be given to studying the genetic association between single nucleotide polymorphisms (SNPs) and disease-related traits (Al-Habib et al., 2020; Dodgson et al., 2000). Therefore, the present study was planned to identify the SNPs in *Mx1* and *ROBO2* gene associated with antibody response to Avian influenza (H9N2) and Newcastle disease viruses respectively in Aseel chicken.

## MATERIAL AND METHODS

### Ethical statement

Ethical permission for the collection of blood samples was obtained with following number DR/639 from the Ethical Review Committee (ERC) for animal research of University of Veterinary and Animal Sciences (UVAS), Lahore, Pakistan.

### Experimental population and blood sampling

The birds used in this study were of adult (45wk old) age and belonged to a closed breeding population of Aseel birds, experiencing family selection, maintained at Indigenous Chicken Genetic Resource Centre, UVAS, Ravi Campus, Pattoki. The birds were kept in automatic breeding cages, manufactured by FACCO (ITALY), and fed commercial breeder ration and managed under a lighting regime of 16:8hr light to dark ratio. Blood samples with a volume of 1.5 ml were collected aseptically from wing vein of each of 500 adult Aseel birds with a sterile syringe (3ml) fitted to 22guage needle. Finally, each blood sample was poured into a separate and sterile 15ml falcon tube containing no anticoagulant. As these birds were of adult age and, therefore, were vaccinated several times against ND and AI, before the start of this experiment. Before performing the sampling, the birds were not vaccinated for more than 3 months. In this study, two blood sampling was performed from each bird. First (pre-vaccination) blood sampling was performed one week before vaccination and second (post-vaccination) blood sampling was performed after two weeks of vaccination of the chickens against AIV (H9N2) and NDV. For vaccination purpose, each bird was intramuscularly injected with 0.3 ml of killed vaccine of ND and H9N2 (Gallimune$^{(TM)}$, manufactured by Merial, Germany) by following the manufacturer's recommendations. Collected blood samples were allowed to stay at room temperature for 30 minutes to enable them to form a clot. The clotted blood samples were then centrifuged at 5000 rpm for 5 minutes at 4°C using the centrifuge machine (Hettich® Universal 320R, Germany) to extract serum. And the remaining blood cells were stored at -20ºC for subsequent DNA extraction. Serum samples were stored at -80ºC before performing Hemagglutination Inhibition (HI) tests.

### Hemagglutination & Hemagglutination Inhibition (HI) Tests

Hemagglutination tests were performed to get 4HA of AI (H9N2) and ND viruses, separately, to perform Hemagglutination Inhibition (HI) tests. The test was performed by following the protocol as described by Allan et al. (1974). Antibody titers in serum samples of first and second sampling were tested through standard HI tests against both Avian Influenza (H9N2) and Newcastle disease viruses separately (Fig.1 and Fig. 2), by the same person. The HI tests were performed for 500 serum samples against H9N2 strain of AIV and 500 serum samples against the NDV separately. In pre-vaccinated samples, serum samples were not diluted for HI tests but in case of post-vaccinated samples, serum samples were used with 10-fold dilution for HI tests. All of the pre- and post-vaccination samples were subjected to HI test in comparison with their positive and negative controls. Then on the basis of maximum divergence in their titer values, the samples were categorized as the low (Group 1) and high titer group (Group 2) for both AIV and NDV, separately. In case of Avian Influenza, the geometric mean (GM) values of pre- and post-vaccination titers for the low antibody group (G1) were 3 $\log_2$ and 9 $\log_2$ respectively. Likewise, the GM values for pre- and post-vaccination titer values for the high antibody response group (G2) were 10 $\log_2$ and 14 $\log_2$ respectively. However, in case of ND the GMT values for pre- and post-vaccination titer values for the low antibody response group (G1) were 8 $\log_2$ and 10 $\log_2$, respectively. And the GM values for pre- and post-vaccination titre values for high antibody response group (G2) were 11 $\log_2$ and 13 $\log_2$ respectively. In total forty birds were selected for AIV, twenty for each of Group 1 and Group 2 on the basis of the above-mentioned criteria. Likewise, in case of Newcastle disease 20 birds were selected for Group 1 and 20 birds for Group 2 on the basis of their antibody response to NDV.

**DNA extraction**

Genomic DNA was extracted from the blood cells of selected samples by using a modified method of Sambrook et al. (1989) and the extracted DNA samples were stored at -20ºC, for further usage, after confirmation through 1% agarose gel electrophoresis by using the standard protocol.

**Primer Designing**

On the basis of the published literature regarding the polymorphisms in *MX1* gene (Ko et al., 2004) and *ROBO2* gene (Luo et al., 2013; Wang et al., 2014) associated with antibody response to AIV and ND, respectively, in chicken, the PCR primers for the amplification of exon-14 of *Mx1* gene (Ko et al., 2004) and 5'UTR & Intron-7 of the *ROBO2* gene (Luo et al., 2013) of Aseel chicken were designed. For primer designing the Primer3 software was used after retrieving the DNA sequences of these genes from the UCSC genome browser (https://genome.ucsc.edu/) and designed primer pairs were synthesized commercially. The details of primers pairs with their chromosomal positions, based on *Gallus_gallus-5.0/galGal5*, are given below in Table 1.

**PCR amplification and confirmation**

Polymerase chain reaction (PCR) was carried out in a final reaction volume of 20µL in ProFlex PCR system (ThermoFisher, USA). The reaction mixture consisted of 0.2 mM dNTPs, 1.2 mM $MgCl_2$, 1X PCR buffer, 100pmol of each of forward and reverse primers, 1 unit of Taq DNA polymerase, and 50ng of template DNA. Polymerase chain reaction was performed under the following protocol; initial denaturation was performed only once at 95ºC for 5min and each of the subsequent 30 cycles was comprised of denaturation for 30 seconds at 94ºC, annealing for 45 seconds at 58-60$^0$C for respective primer pairs (Table 1), and extension at 72 ºC for 45 seconds. The final extension was performed for 10 minutes at 72ºC. Finally, the amplified PCR products were subjected to 1.2% agarose gel electrophoresis along with 50bp DNA ladder and bands were visualized under UV light in BIO-RAD gel documentation system (California, USA).

**DNA Sequencing of amplified PCR products**

PCR products were purified and Sanger sequenced by using the Genetic Analyzer (ABI-3130) and the sequence data were aligned by using CLUSTALW algorithm in MEGA-7 software (Kumar et al., 2018). Polymorphisms in the sequence data were determined by using the DnaSP software (http://www.ub.edu/dnasp/).

**Data Analysis**

The GenAlEx software (v6.503) was used to calculate the allelic and genotypic frequencies, along with expected number of alleles (Ne), and Chi-square value for the DNA sequence data.

For the association analysis, the "One-Way Analysis of Variance" procedure of GenStat (version 19.1) was used to analyze the data by considering the genotype as the fixed effect. Following statistical model was used for the data analysis

$$Y_{ij} = \mu + G_i + e_{ij}$$

Where Y= Dependent variable,
µ= Population mean,
G= Fixed effect of the genotype, where i = [1, 2, & 3]
e= Residual error.

## RESULTS

**PCR amplification**

The amplified PCR products of exon-14 of *Mx1* gene showed a size of 642bp (Fig. 1), which was in agreement with the position of designed primers. Likewise, the amplified PCR products of Intron-7 and 5'UTR of *ROBO2* gene showed a size of 660bp and 686bp respectively (Fig. 2, & 3) showing the primer-specific amplifications.

**Table 1** Primer sets along with their amplicon size and annealing temperatures

| Gene symbol | Forward and Reverse Primers sequences | Starting position (bp) on Chr. 1 | Amplicon Size (bp) | Annealing Temp.($^0$C) | Gene Region |
|---|---|---|---|---|---|
| Mx1 | F:AGCAACTCCATACCGTGTTTT<br>R:GCTCCCCCTCCTTTCAAATA | 108941442<br>108942064 | 642 | 58 | Exon 14 |
| ROBO2 | F:CACTTTGAGTTATGTTTCAAGGTTTG<br>R:CAAAGGTGCAATGGTTTTACC | 97000466<br>97001105 | 660 | 60 | Intron 7 |
| ROBO2 | F:CCCTCTGAATGGCATCTGTT<br>R:GCCATTTCTAATACATCATGGAGA | 97589863<br>97590525 | 686 | 59 | 5'UTR |

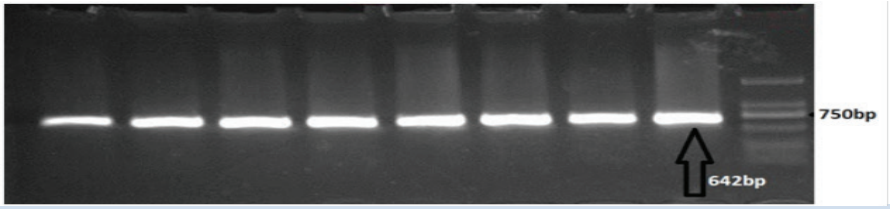


**Figure 1** Gel image of PCR products (642bp) of exon-14 of Mx1 gene

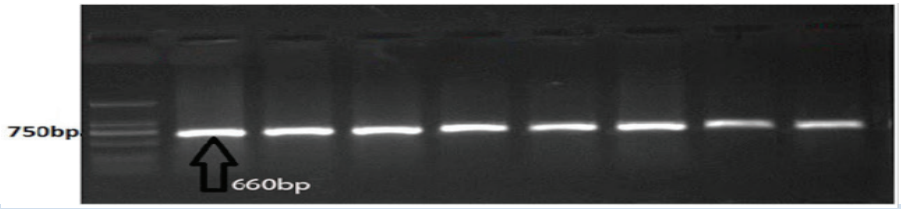


**Figure 2** Gel image of PCR products (660bp) of Intron-7 of ROBO2 gene

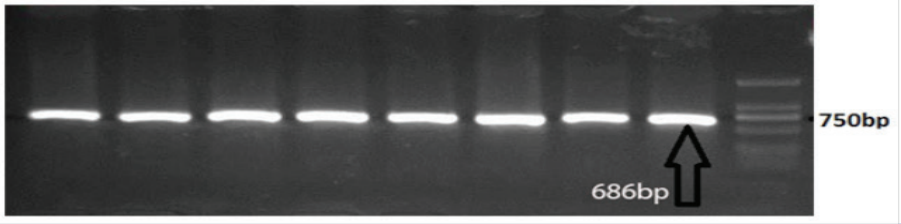


**Figure 3** Gel image of PCR products (686bp) of 5'UTR of ROBO2 gene

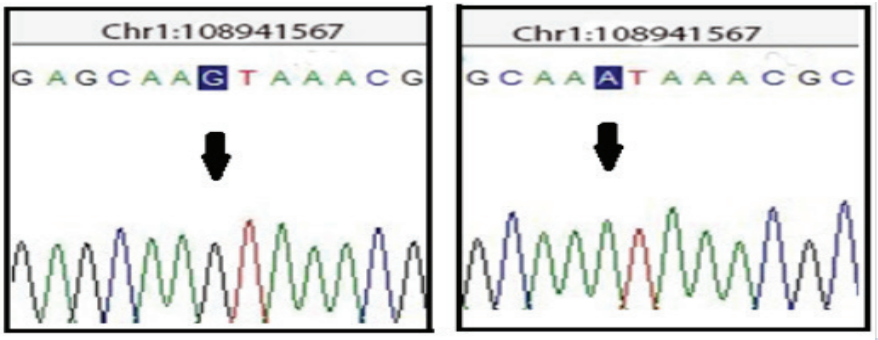


**Figure 4** Electropherogram of nucleotide sequence representing nucleotide substitution (G>A) at Chr1:108941567bp in exon 14 of Mx1 gene in Aseel chicken. The changed nucleotides are highlighted blue and are represented by downward arrows.

**Identification of single nucleotide polymorphisms in *Mx1* (Exon14) and *ROBO2* (Intron 7 and 5'UTR) gene**

Sequence data of exon-14 of *Mx1* gene revealed three single nucleotide polymorphisms in Aseel chickens (Table 2 & 3). The first variant (G>A) was observed at Chr1:108941567 bp (Figure 4), second (G>A) at Chr1:108941793 bp, and third (A>G) at Chr1:108941810bp. The SNP Chr1:108941567 bp was proved non-synonymous and the wild-type allele (G) was substituted with Adenine (A), changing AGT codon into AAT which led to the change of amino acid Serine (S) to Asparagine (N) at position 631 in the Mx protein. Likewise, A>G polymorphism at Chr1:108941810bp was also non-synonymous mutation causing a change of amino acid Lysine>Arginine at position 712 in the Mx protein. The allelic and genotypic frequencies of the single nucleotide variants observed in exon 14 of the *Mx1* gene and intron-7 and 5'UTR of *ROBO2* genes along with their Number of expected alleles (Ne), and Chi-square values are given in Table 2.

**Single nucleotide polymorphisms in *ROBO2* gene (Intron 7 and 5'UTR)**

Sequencing data of Intron 7 of *ROBO2* gene showed two single nucleotide polymorphisms in Aseel chickens. The first variant (C>T) was at Chr1:97000797bp,

and second (T>A) at Chr1: 97001015bp (Table 3). However, the sequence data of 5'UTR of *ROBO2* gene showed one single nucleotide variant (T>C) (Table 3) at Chr1:97589984bp. The allelic and genotypic frequencies of the single nucleotide variants observed in intron 7 and 5'UTR of *ROBO2* genes along with their Number of expected alleles (Ne), and Chi-square values are given in Table 2.

**Association analysis of SNPs in *Mx1*-exon14 gene with antibody response against AIV**

All three SNPs found in exon 14 of *Mx1* gene were tested for their association with pre- and post-vaccination antibody response of Aseel birds against AIV (H9N2). However, only one SNP (Mx1_567) was found significantly ($P<0.01$) associated with pre- and post-vaccination ($P<0.01$) antibody response (Table 4). This substitution SNP (G>C) at 8 bp of exon 14 was a non-synonymous polymorphism causing a change of amino acid Serine by Asparagine at 631 bp position in Mx1 protein.

**Association analysis of SNPs in *ROBO2* Gene (Intron-7 and 5'UTR) with antibody response against ND virus**

The two SNPs found in intron 7 and one in 5'UTR of *ROBO2* gene were tested for their association with antibody response against NDV. However, no SNP in *ROBO2* gene was found significantly associated with pre- and post-vaccination antibody response of Aseel chicken against NDV.

**Table 2** Allelic and genotypic frequency of variations in exon 14 of Mx1 gene and in Intron 7 and 5'UTR of ROBO2 gene

| Gene | Locus Name | | Genotypes and alleles | Frequency | Ne | $X^2$ |
|---|---|---|---|---|---|---|
| Mx1 | Mx1_ 567 | Genotypes | AA | 0.306 | 1.824 | 0.746$^{ns}$ |
| | | | AG | 0.417 | | |
| | | | GG | 0.278 | | |
| | | Alleles | A | 0.51 | | |
| | | | G | 0.49 | | |
| | Mx1_ 793 | Genotypes | GG | 0.861 | 1.147 | 0.066$^{ns}$ |
| | | | GA | 0.139 | | |
| | | | AA | 0.000 | | |
| | | Alleles | G | 0.861 | | |
| | | | A | 0.139 | | |
| | Mx1_ 810 | Genotypes | AA | 0.000 | 1.944 | 10.956$^{**}$ |
| | | | AG | 0.833 | | |
| | | | GG | 0.167 | | |
| | | Alleles | A | 0.833 | | |
| | | | G | 0.167 | | |
| ROBO2 | ROBO2_1_797 | Genotypes | CC | 0.424 | 1.730 | 0.729$^{ns}$ |
| | | | CT | 0.484 | | |
| | | | TT | 0.092 | | |
| | | Alleles | C | 0.666 | | |
| | | | T | 0.334 | | |
| | ROBO2_1_015 | Genotypes | TT | 0.707 | 1.333 | 0.325$^{ns}$ |
| | | | TA | 0.303 | | |
| | | | AA | 0.00 | | |
| | | Alleles | T | 0.727 | | |
| | | | A | 0.273 | | |
| | ROBO2_2_984 | Genotypes | TT | 0.879 | 1.132 | 0.809$^{ns}$ |
| | | | TC | 0.121 | | |
| | | | CC | 0.000 | | |
| | | Alleles | T | 0.909 | | |
| | | | C | 0.091 | | |

NS Non-significant
** P<0.01

## DISCUSSION

In the current study, the investigation was carried out to find out the genetic variability in the form of single nucleotide polymorphism (SNP) in exon-14 of *Mx1* gene and in intron-7 and 5'UTR of *ROBO2* gene associated with antibody response to AIV and NDV respectively, in Aseel chickens. The sequence data of exon-14 of *Mx1* gene showed three single nucleotide variants, whereas 2 of them were non-synonymous SNPs (Table 3), and likewise the sequence data of the selected region of *ROBO2* gene revealed 3 SNPs (Table 2). The population genetic parameters including allelic and genotypic frequencies of these variants along with the Number of expected alleles (Ne), and Chi-square values for each of these variants found in *Mx1* and *ROBO2* genes were calculated in order to determine the goodness-of-fit (Table 2). However, all the loci showed non-significant differences in the genotypic frequencies suggesting that they were under Hardy-Weinberg equilibrium except Mx1_810 which showed significant ($P<0.01$) differences.

In the case of antibody response to AIV the titer ranged from 3 $\log_2$ to 14 $\log_2$ and in the case of ND it ranged from 8 $\log_2$ to 13 $\log_2$ indicating the genetic variability in antibody response to both diseases in Aseel chickens which could be used to explore the underlying genetic variations (Psifidi et al., 2016; Zhang L et al., 2015). The present study determined the association of single nucleotide polymorphisms in *Mx1* gene with pre- and post-vaccination antibody response to Avian Influenza (H9N2) virus in Aseel chickens. The results of association analysis showed that one variant (G>A) at 108941567 bp position (8$^{th}$ bp of exon 14) was significantly associated with pre- ($P<0.01$) and post-vaccination ($P<0.01$) antibody response against AIV (H9N2). It was also observed that this non-synonymous single nucleotide substitution was changing amino acid Serine (AGT) into Asparagine (AAT) at 631$^{st}$position in Mx protein. In agreement with our results the same variant (Guanine substituted with Adenine) at 8 bp of exon-14 had also been reported to be associated with antibody response against Avian influenza A virus in chickens by some other authors (Ko et al., 2004; Sironi et al., 2008).

Moreover, several other authors had also been reported that the presence of non-synonymous SNP in exon-14 of *Mx1* gene and subsequent change of amino acid Serine with Asparagine at 631$^{st}$position in Mx1 protein molecule made this protein highly anti-viral against Avian influenza virus in different chicken breeds (Ewald et al., 2011; Ko et al., 2002; Sartika et al., 2011; Seyama et al., 2006) which was initially known to lack any anti-viral activity (Ko et al., 2004). We suggest that the identification of additional candidate genes independently contributing resistance to AIV in chickens would be necessary to achieve strong genetic resistance by selection. Likewise, the ISG15 (Lenschow et al., 2007) and interferon genes (Koerner et al., 2006) had also been implicated as critical in protection against influenza in mice. In a similar study, Grimm et al. (2007) observed that the inoculation of influenza virus resulted in killing of mice within a few days irrespective of the presence of amino acid at 631$^{st}$ position in its protein. However, the pre-treatment of the mice with type-I interferon before the challenge of influenza virus resulted in survival of those mice having Asparagine amino acid at 631$^{st}$ position, whereas those with Serine at that position were all died (Grimm et al., 2007). These results indicated the importance of type-I interferon in protection against a fatal influenza virus but also demonstrate that a functional Mx1 protein is crucial for the type-I interferon to induce an effective antiviral state. Hence, it is likely that Mx1_567 led resistance to AIV is mediated by type-I and type-II interferons genes which might be in linkage disequilibrium with this SNP because they are also located at chromosome 1, but it merits further investigations.

**Table 3** List of identified variants, their alleles, chromosomal positions and subsequent codons in Mx1 and ROBO2 gene

| Chromosome | Chromosomal Position (bp) | Gene Region | Variant ID | N. and N. change | Codon and change in AA |
|---|---|---|---|---|---|
| 1 | Chr1:108941567 | Exon 14 | Mx1_567 | G>A | AGT>AAT:P. Ser>Asn |
| 1 | Chr1:108941793 | Exon 14 | Mx1_793 | G>A | - |
| 1 | Chr1:108941810 | Exon 14 | Mx1_810 | A>G | AAA>AGA:P. Lys>Arg |
| 1 | Chr1:97000797 | Intron 7 | ROBO2_1_797 | C>T | - |
| 1 | Chr1: 97001015 | Intron 7 | ROBO2_1_015 | T>A | - |
| 1 | Chr1:97589984 | 5'UTR | ROBO2_2_984 | T>C | - |

Positions are based on 5$^{th}$ genome assembly of chicken genome (Gallus_gallus-5.0/galGal5), Dec 2015, available at UCSC genome browser (http://genome.ucsc.edu).

**Table 4** Least square mean values ($log_2$) of titer against AIV for different genotypes at 108941567bp locus of Mx1 gene

| Antigen/virus | Gene | Locus (bp) | Genotypes | Pre-vaccination titer | Post-vaccination titer |
|---|---|---|---|---|---|
| AIV (H9N2) | Mx1 | 108941567 | AA | 8.21±1.23[a] | 13.86±1.54[a] |
| | | | AG | 7.20±1.19[a] | 13.00±1.50[a] |
| | | | GG | 4.00±1.13[b] | 8.55±1.42[b] |

Values in the same column but with different superscript vary significantly

Additionally, there is also a need to explore and identify different canonical pathways in order to understand how conditioning with interferon is involved in making the mice with Asparagine at 631 amino acid position in Mx1 protein survive upon exposure to influenza virus. Moreover, the results of least significant difference showed that homozygous and heterozygous mutant (AA, and AG) genotypes were associated with significantly greater antibody response compared with wild-type (GG) genotype (Table 4). However, the titer values for AG and AA were the same suggesting the completely dominant mode of action of mutant allele (A). Though this SNP had been studied in several chicken breeds but has never been reported in Aseel chickens before. It is likely that selective breeding of superior birds based on this polymorphism might be valuable in achieving a better immune response against AIV in Aseel chickens.

The present study also determined the association of single nucleotide polymorphisms in *ROBO2* gene (Intron-7 and 5'UTR) with antibody response to NDV in Aseel chickens because *ROBO2* is known to have its role in regulation of chicken immune response to NDV (Luo et al., 2013; Wang et al., 2013). However, none of the SNP observed in the intron-7 and 5UTR of *ROBO2* gene was found significantly associated with $log_2$ values of antibody response against ND. These results are not compatible with the findings of Luo et al. (2013) who observed that SNP at 97000797 bp in intron-7 of *ROBO2* gene was found associated with antibody response to NDV, but in the current study though that a SNP was observed but was found non-significant. The likely reason for its non-significant effects could be a smaller sample size in the current study, and it is likely that increase in sample size could result in a significant association of that SNP with anti-ND antibody response. The other possibility for the non-significance of Chr1:97000797 bp in the present study could be the breed differences because Luo et al. (2013) used commercial broilers in their study, whereas the current study is based on Aseel birds. To the best of our knowledge, this is the first study in which association of single nucleotide variant in *Mx1* gene is identified with antibody response to avian influenza virus in Aseel chickens.

## CONCLUDING REMARKS

1. One single nucleotide variant G>A at 108941567 bp in exon 14 of *Mx1* gene was found significantly associated with pre- ($P<0.01$) and post-vaccination ($P<0.01$) antibody response against Avian influenza (H9N2) virus in Aseel chicken.

2. It is likely that the identified SNP could be used for a better understanding of the biology of immune response to AI and to select the Aseel birds with increased antibody response against AI.

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## COMPETING INTERESTS

The authors declare that they have no competing interests.

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