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Molecular Characterization of the *NRAMP1* Gene and Blood Parameters of Sinai and Lohman Brown Chickens in Egypt

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ABSTRACT

In almost all animal species, natural resistance-associated macrophage proteins (*NRAMPs*) have been linked to disease resistance. It plays a crucial part in innate immunity and can affect adaptive immunity as well. The aim of this study was to investigate some immunological traits and molecular genetics in the native breed of chickens, named Sinai (S) and a commercial strain of Lohman Brown (LB). The *NRAMP1* gene was reported to be associated with a defense mechanism against infection by bacteria and viruses. A total of 144 female day-old chicks, including 72 from the commercial layer strain (LB) and 72 from the Egyptian native chicken strain (S), were used in this study. At 38 days of age, blood samples were taken randomly from 8 chickens of each group for serum antibodies against the New Castle disease virus, avian influenza virus, and infectious bursal disease virus analysis. Additionally, genomic DNA was extracted from 20 blood samples at 38 days of age. Polymerase chain reaction (PCR) analyses were conducted on the DNA samples, followed by sequencing of the PCR products to identify single nucleotide polymorphisms (SNPs) in the *NRAMP1* gene in the two strains of chickens. The findings indicated that lymphocyte, eosinophil, phagocyte activity, and IgY were significantly greater in LB chicks than in S chicks. Sinai chickens, on the other hand, achieved dominance in Newcastle titter. Eight SNPs were found in *NRAMP1* of the two strains. The nucleotide identity between S and LB nucleotides was 58.68%, while the changes in different amino acids were found in different positions. Multiple SNPs in the *NRAMP1* gene have been discovered in Sinai and LB, suggesting that this gene can be used as a genetic marker for the selection of high-producing indigenous hybrids with the ability to resist pathogenic diseases in poultry.

Keywords: Disease resistance, Lohman Brown, Sinai, Gene, Single Nucleotide Polymorphism

INTRODUCTION

Poultry plays a major role in the rural economy as a source of animal protein and as a contributor to income since its products are relatively inexpensive and widely available. In an attempt to increase the productivity of poultry, numerous breeding programs have been developed, and the current breeding programs that enhance poultry resistance to diseases have grown in popularity as an alternate strategy for increasing productivity while lowering production costs (FAO, 2013). The importance of native chickens for the rural economy and backyard farming is immense in some countries (Agarwal et al., 2020). Local chickens are important in contributing to the food security of rural households across developing countries. Indigenous chickens in Africa account for 80% of all chickens (Goodger et al., 2002).

The Sinai chicken breed was raised in the desert areas of the governorates of North and South Sinai (Gebriel et al., 2018). Local chicken farms often face low disease resistance (Pagala et al., 2013). The Sinai breed comes from the natural mating of some foreign breeds with local Egyptian chickens. In Egypt, this breed is used as egg-type chicken. Numerous studies have revealed that genetic factors significantly determine disease vulnerability and resistance in chickens (Saleh et al., 2020; 2021). Lohman Brown (LB) is a

commercial chicken breed that was created in Germany. It is known for its good egg-laying ability, high production rate, and the ability to adapt to a different environment.

Natural resistance-associated macrophage protein 1 (*NRAMP1*) is a protein that plays a crucial role in eliminating iron ions from macrophages. It is abundantly present in cells and exhibits upregulation following pathogen infections in various animal species (Soe-Lin et al., 2009; Tohidi et al., 2013). Numerous animal species have the functional candidate *NRAMP1* gene, which exhibits disease-resistance action (Lamont et al., 2002). The *NRAMP1* gene is linked to the characteristics of chicken immunity (Hu et al., 2011). On chicken chromosome 7, a homolog of *NRAMP1* has been located. It has a promoter region, 15 exons, 14 introns, and flanking regions that total 5760 bp in length (Desmond et al., 2019). Advances in molecular technology have created a new horizon for the genetic improvement of traits in poultry (Salah et al., 2021).

A single nucleotide polymorphism (SNP) is the most common type of genetic variation in the genome (Eichler et al., 2007). Two types of SNPs are found in coding sequences, synonymous (cause no changes to an amino acid) and nonsynonymous. Nonsynonymous SNPs are interesting because they might influence how proteins are expressed. Conversely, synonymous SNPs most likely have no impact on gene expression. For mapping investigations, synonymous and nonsynonymous SNPs are excellent genetic markers (Emara and Kim, 2003). According to Ardiyana et al. (2020), Newcastle disease antibody titers in SenSi-1 Agrinak chickens were substantially correlated with the TC genotype of the *NRAMP-1* gene. The present study aimed to screen SNPs within the *NRAMP1* gene of indigenous and exotic chicken strains. Additionally, immunological investigations between the two strains were also conducted.

MATERIALS AND METHODS

Ethical approval

Animal and Poultry Production Scientific and Ethics Committee, Faculty of Agriculture, Damanhour University, Egypt (DUFA-2020-11) authorized all experimental techniques.

Study animals

The study started in December 2020 until December 2021 at the Animal and Poultry Research Farm, El-Bostan, and genetic engineering laboratory, Damanhour University, Egypt. A total of 144 female day-old chicks, including 72 from the commercial layer strain (Lohman Brown) and 72 Egyptian native chickens from the Sinai strain, were used in this study. Chicks were wing banded at hatching and randomly divided into two groups with similar initial body weights in battery brooders (35 cm [L] × 25 cm [W] × 30 cm [H]) from day 1 to day 38 of age. There were 12 replicates per strain, with 6 chickens per replicate. Hitchiner B1 (LAPROVET, France) + Gumboro (Zoetis, USA), Influenza H5 N2 (Zoetis, USA), Colon30 (LAPROVET, France), Gumboro 123 (Zoetis, USA), Colon79 (LAPROVET, France), and Lasota (LAPROVET, France) vaccinations were given on days 7, 9, 10, 16, 20 and 30, respectively. A 23:1 light/dark cycle was used from day 2 until day 38 of the experiment, with the ambient temperature and relative humidity being 32.4 ± 4°C and 46.9 ± 6%, respectively, and distributed to battery brooders. Until 3 weeks of age, all chicks were fed a commercial diet containing 23% crude protein and 3040 kcal/kg of ME and were fed *ad libitum*. They were fed a diet with 21% crude protein and 3102 kcal/kg until they were 6 weeks old.

At 38 days of age, approximately 3 ml blood samples were taken randomly from the wing vein of 8 chickens from each strain. Two sterile centrifuge tubes, one with and one without EDTA as an anticoagulant, were used to collect the blood samples. A tube with EDTA was used to measure blood hematology, including white blood cell count (WBC), differentiation of WBC, red blood cell count (RBC), hemoglobin, packed cell volume (PCV), phagocytic activity (PA), and phagocytic index (PI). The tubes were centrifuged for 20 minutes at 3,000 rpm to clearly separate the serum from the plasma, and then the serum and plasma were kept at -20°C. Plasma total protein (g/100 ml) was measured according to Weichselbaum (1946). The albumin concentration (g/100 ml) was determined according to the method of Dumas et al. (1997). The globulin concentration (g/100 ml) was estimated by subtraction of the albumin concentration from the plasma total protein value according to Coles (1986). Serum immunoglobulin (IgY, IgM, and IgA) was determined as described previously by ELnaggar et al. (2016). Blood hematology (Table 2) was performed as indicated by Attia et al. (2014). According to Kawahara et al. (1991), phagocytic activity and phagocytic index were also determined. The hemagglutination inhibition (HI) test was used to detect serum antibodies against Newcastle disease virus (NDV) and avian influenza virus (AIV), according to King and Seal (1998) and Takátsy (1955), respectively. The antibody titer for the infectious bursal disease virus (IBDV) was determined using an infectious bursal disease virus antibody test kit (HIBD-001, China).

DNA amplification

A total of 20 chickens at 38 days old from each strain had blood drawn into sterile, EDTA-treated collecting tubes. Whole blood genomic DNA purification mini kit (Thermo Scientific, Gene JET, Germany) was used to isolate genomic DNA from whole blood. DNA samples were analyzed on a spectrophotometer (T80, UK) to determine their concentration and purity. For PCR experiments, approximately 100 ng of genomic DNA from the pool of each strain was used as a PCR template. 20 ul of distilled water, 10 ul of PCR master mix (Fast gene taq ready mix, lot No. LS 27-192708, Germany), and 1 ul of each primer (F, R) were used for the amplification. The PCR reactions were subjected to amplification using a thermal cycler (Bio-Rad, USA) under the following cycling conditions, an initial denaturation step at 95°C for 5 minutes, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 66 °C for 30 seconds, and extension at 72 °C for 30 seconds. The ultimate elongation was executed at a temperature of 72 °C for 5 minutes. The electrophoretic analysis of the PCR product was conducted on a 1.5% agarose gel at 100 volts for a period of 35 minutes. The visualization of the product was accomplished through the utilization of a gel documentation system manufactured by Bio-Rad (Gel Doc EZ System, USA). Table 1 indicates the National Center for Biotechnology Information (NCBI) accession number, forward and reverse primers, as well as amplicon sizes utilized in the present study. Primers were designed using Primer-BLAST from the database sequence of GenBank accession. This primer was obtained from Invitrogen, Thermofisher Scientific, UK.

Table 1. The primer sequence used for amplification of the coding region of *NRAMP1* gene

Gene name	NCBI	Amplification size	Sequence
NRAMP1	AY072001	421 bp	Forward 5`CAATGAGACGGTGTCTGTGG3`
			Reverse 5` CCCAGAAGAAATCTCCCTGC3`

NCBI: National Center for Biotechnology Information; *NRAMP1*: Natural resistance-associated macrophage protein 1

PCR sequencing and processing

The PCR products were purified using a PCR purification kit (QIAquick, Qiagen, Germany). The sequence was carried out using a DNA automated sequencer. Many amplicons from the *NRAMP*1 gene were chosen and sequenced in one way (forward primer). The sequence results were analyzed using Chromas 1.45 software. The obtained sequences were aligned using Clustal Omega (Sievers et al., 2011). The amino acid translation was performed using EXPASY-Translate tools (Gasteiger et al., 2003).

Statistical analysis

The statistical analysis was performed using the general linear model (GLM) using SAS (2016). The statistical model used in this study was according to Formula 1.

$$Y_{ij}= \mu+S_i+e_{ij} \quad \text{(Formula 1)}$$

Where, Y_{ij} is the observation, μ denotes the general mean, S_i signifies the fixed effect of the strain *(i)*, and e_{ij} determines the random error. Tukey's test was utilized to evaluate the statistical significance between means at a significance level of p less than 0.05.

RESULTS

The data in Table 2 show the effect of strain on blood hematology in chicks. The results showed that the percentage of lymphocytes, the percentage of eosinophils, the percentage of heterophils, the ratio of heterophils to lymphocytes, and the phagocyte activity were all significantly affected by strain. The results showed that the lymphocyte ($p < 0.05$), eosinophil ($p<0.05$), and phagocyte activities ($p < 0.05$) of LB chicks were significantly greater than those of Sinai chicks. On the other hand, the heterophil-to-lymphocyte ratio of Sinai chicks was higher than those of LB chicks ($p < 0.05$). Table 3 shows the difference in blood biochemistry between the two strains. Sinai chicks had higher albumin and hemagglutination inhibition antibody against Newcastle disease virus (HINDV) compared to the Lohman strain. The opposite trend was observed for the immunoglobulin yolk (IgY) concentration, where the LB chicks had a significantly higher IgY concentration than the indigenous chicks. Meanwhile, there was no statistically significant difference between strains in hemagglutination inhibition antibody against avian influenza disease virus and hemagglutination inhibition antibody for infectious bursal disease virus.

***NRAMP1* gene polymorphism**

The *NRAMP1* gene fragment size after amplification was 421 bp. The *NRAMP1* gene was discovered to be nonpolymorphic across all chicken strains, and only one allele form was found. Sequence alignment of the *NRAMP1* gene between the Sinai and LB strains revealed the presence of repeated SNPs at various positions (Figure 1). The results reveal 58.68% identity for nucleotides between Sinai and LB chicks by Clustal Omega and 99% by NCBI in this gene, as shown in Table 4.

In the current study, eight SNPs were identified in *NRAMP1*. The translation of chicken *NRAMP1* sequencing in Sinai and LB is illustrated in Figure 2. At position 1bp nucleotide transition (A ↔ C), 6 bp (A ↔ G), 10 bp (T ↔ G), 11 bp (G ↔ A), and 12 bp (A ↔ G) were observed. Moreover, at position 13 bp, 14 bp (T ↔ G), and 162 bp (T ↔ C) were seen.

Present results revealed that the percent identity for amino acids were 88.30%. A change in amino acid number 84-85 from arginine to glycine (84-85 R > G) and amino acid number 86 from threonine to glycine (86 T > G), amino acid number 87 from serine to glutamic acid (87 R > G), amino acid number 88 from histidine to alanine (88 H > A), and amino acid number 89 from histidine to glycine (89 H > G). Moreover, a change in amino acid number 90 from aspartic acid to lysine (90 D > K), amino acid 91 from alanine to tryptophan (91 A > W), amino acid 92 from alanine to histidine (92 A > H), amino acid 93 from arginine to valine (93 A > V), and amino acid number 94 from alanine to arginine (94 A > R) are illustrated in Figure 3.

Table 2. Hematological parameters of Sinai and Lohman Brown chicks at 38 days of age in Egypt

Variable	Lohman Brown	Sinai	p-value	SEM
WBC (10^3/mm3)	19.50	20.57	0.107	0.454
Lymphocyte (%)	42.43[a]	40.50[b]	0.009	0.483
Monocyte (%)	11.79	11.21	0.189	0.299
Basophils (%)	0.714	0.357	0.061	0.226
Eosinophils (%)	11.57[a]	10.28[b]	0.002	0.258
Heterophils (%)	33.50[b]	37.64[a]	0.0001	0.608
Heterophil to lymphocyte ratio	0.789[b]	0.929[a]	0.0009	0.029
Phagocytic Activity	20.86[a]	19.71[b]	0.020	0.325
Phagocytic Index	1.730	1.650	0.285	0.051
RBC (10^6/mm3)	1.490	1.520	0.665	0.046
Hemoglobin (g/dl)	10.78	10.93	0.626	0.205
PCV (%)	32.00	32.21	0.796	0.580
MCH (pg)	73.25	72.65	0.876	2.690
MCHC (g/dl)	33.73	33.94	0.718	0.414
MCV (mm3)	217.7	214.1	0.758	8.230

SEM: Standard error of the mean, WBC: White blood cell, RBC: Red blood cell, PCV: Packed cell volume, MCH: Mean corpuscular hemoglobin, MCHC: Mean corpuscular hemoglobin concentration, MCV: Mean corpuscular volume. [ab] Means with different letters in a row differ significantly ($p < 0.05$).

Table 3. Effect of chicken strain on blood immunological characteristics in Egypt

Traits	Lohman Brown	Sinai	p-value	SEM
Total protein	5.58	5.74	0.187	0.082
Albumin	3.31[b]	3.53[a]	0.003	0.049
Globulin	2.28	2.21	0.6145	0.099
IgM	234.0	236.5	0.1181	1.09
IgY	977.1[a]	968.2[b]	0.002	1.79
HINDV	3.71[b]	4.21[a]	0.035	0.025
HIIBD	3.07	2.64	0.209	0.055
HIAI	1.50	1.71	0.452	0.039

SEM: Standard error of the mean, IgA: Immunoglobulin A, IgM: Immunoglobulin M, IgY: Immunoglobulin Y, HINDV: Hemagglutination inhibition antibody against Newcastle disease virus, HIIBD: Hemagglutination inhibition antibody against infectious bursa disease virus, HIAI: Hemagglutination inhibition antibody against avian influenza virus [ab] Means with different letters in a row differ significantly ($p < 0.05$).

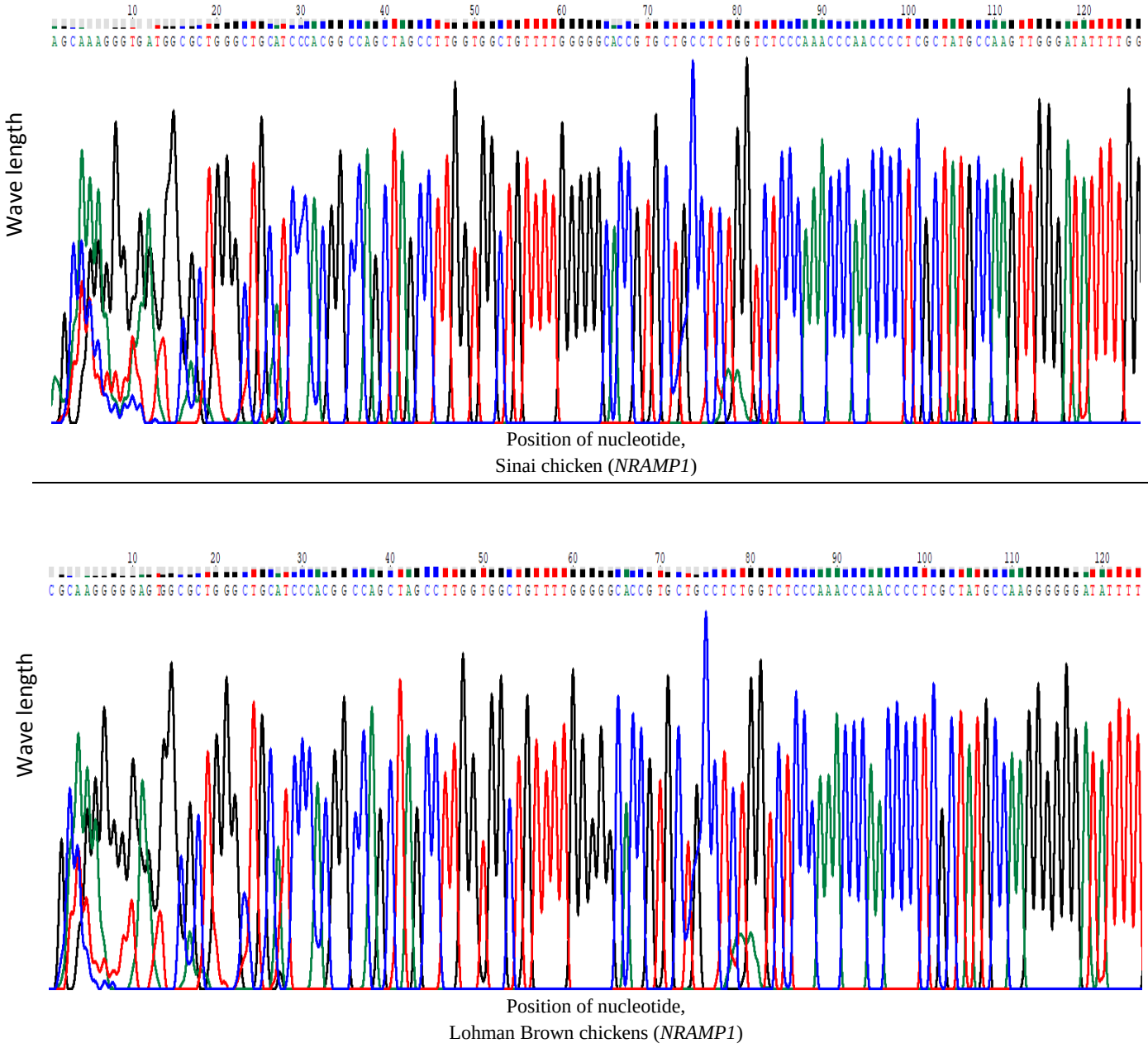

Figure 1. *NRAMP1* gene sequences collected from local Egyptian strain (Sinai) and Lohman Brown chickens in Egypt

Table 4. Percent identity matrix for nucleotide and amino acids in Siani and Lohman Brown chicks in Egypt

Strain	Nucleotide	Amino acids
sp|P69905|*NRAMP1*_Sinai	100.00 58.68	100.00 88.30
sp|P01942|*NRAMP1*_Lohman	58.68 100.00	88.30 100.00

Figure 2. Nucleotide alignment (bp) of *NRAMP1* for Sinai and Lohman Brown strains in Egypt

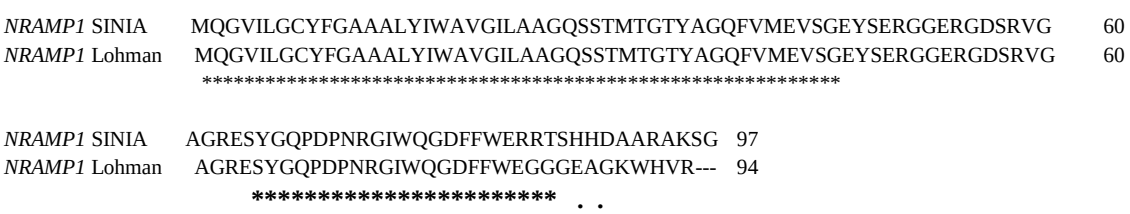

Figure 3. Amino acids alignment (bp) of *NRAMP1* for Sinai and Lohman strains in Egypt

DISCUSSION

In this study, commercial chicks (LB) had a higher lymphocyte and eosinophil percentage than the local chicks. This finding contrasts with the results of El-Safty (2012), who reported that local Libyan chicks had a higher lymphocyte percentage and lower heterophil percentage. According to Ferrante et al. (2016), heterophils increase while lymphocytes decrease when chickens are stressed. Therefore, the ratio of the two parameters is an indicator of stress and infection response. Heterophil and lymphocyte responses to stress have a genetic component (Minias, 2019), and their ratio has been employed as selection criteria for infection resistance in chickens (Thiam et al., 2021). The Egyptian native chicks (Sinai) had a significantly higher H/L ratio than the LB strain. This finding revealed that the Sinai chicks experienced higher stress levels than the LB strains under specific environmental conditions in Egypt. This could be attributed to the origins of the Sinai chicks, which resulted from the natural mating of certain foreign breeds with Egyptian indigenous chickens.

In terms of disease resistance, phagocytic activity is crucial. Chickens with a stronger phagocytic potential and nitrite generation from macrophages may be more resistant to bacterial, viral, and parasite diseases (Galal et al., 2007). It is worth noting that the genetic differences between LB and Egyptian native chicks may have resulted in differing phagocytic responses. Several studies have found that chicken phagocytic activity is genetically controlled (Kundu et al., 2015). The IgY is a maternal antibody passed from progeny through blood serum supplied to egg yolk (Murai, 2013). Both environment (maintenance management, disease exposure) and genetics influence chicken antibody production (Al-Habib et al., 2020). Wibawan et al. (2010) reported that exposure to a

bacterial disease increased IgY concentrations. Moreover, the concentration of IgY in crossbred chickens was higher than in local chickens (Setyawati et al., 2019; Al-Habib et al., 2020). According to the findings of this study, LB chicks had higher IgY concentrations than Sinai chicks under the same maintenance management. The LB chicks are hybrid chicks, and heterosis has a beneficial influence on the immunological response, as indicated by the quick increase in IgY concentration (Al-Habib et al., 2020). Genetics influence the production of Sinai's antibody, as this chicken strain has not been subjected to any artificial selection.

Newcastle disease is a viral disease produced by virulent strains of NDV that affect chickens worldwide (Oberländer et al., 2020). Depending on the viral strain and the host's sensitivity, the virus has a morbidity and death rate of up to 100% (Swayne and Boulianne, 2020). Furthermore, the performance of diseased flocks significantly declines, and infected animals' eggs become thin-shelled. Infections with lentogenic strains of the virus cause modest respiratory symptoms in chickens; however, infections with velogenic strains cause mucous membrane inflammation and central neurological problems in chickens, including torticollis and opisthotonos (Swayne and Boulianne, 2020). The Newcastle disease affects poultry all over the world and specific antibody titers can be used to assess the immunological response. Antibody titers are affected by various factors, including health, the severity of viral infection, and the period of infection (Oberländer et al., 2020). Antibodies are formed 6-10 days after Newcastle infection and reach their peak at 3-4 weeks, after which they decline for up to 3-4 months and are no longer detectable after a year (Anamu and Rohi, 2005).

The local Egyptian strain has a high potential for developing disease-resistant chickens (Kolstad and Abdou, 2000). The genetic potential of disease resistance in local chickens can still be developed using the antibody titer indicator as a selection marker. The selection program can be effectively conducted by implementing optimal vaccination strategies, enabling the identification of the chicken's response to diseases, and facilitating the selection of chickens with superior genetic traits. The superiority of local chickens that are more susceptible to disease agents provides advantages and facilitates genetic improvement in the quality of local Egyptian chickens.

Bioinformatics indicators for variation in allelic frequency at a given locus are provided by genetic markers. Due to the presence of molecular markers in poultry, it is now possible to conduct in-depth investigations and evaluations of genetic diversity and to identify genes impacting economically significant features (Khalil et al., 2021). The *NRAMP1* gene fragment amplified in both native and exotic chicken types were nonpolymorphic, indicating the presence of a single homozygous allele, and the same outcomes were found by Fulton et al. (2014).

The promoter region of the *NRAMP1* gene contains one major and two minor transcription initiation sites, a classical TATAA component, and consensus sequences for the binding of the myeloid-specific PU1 factor, many lipopolysaccharides (NF-IL6 and NF-ardB), and interferon-inducible response components (Marquet et al., 2000). Different genotypes of this gene have been reported to impact the antimicrobial function mediated by microglial cells (Mazzolla et al., 2002). Due to the genetic variability in the *NRAMP1* gene, Girard-Santosuosso et al. (2002) demonstrated that various chicken populations have varying heritability of susceptibility to infection. Tge SNP markers have already been used to identify disease resistance genes in chickens. Additionally, it can be used as an alternative for microsatellite markers in various study areas involving chickens (Emara and Kim, 2003; Malek and Lamont, 2003).

Polymorphism in the *NRAMP1* gene has been linked to a *Salmonella enteritidis* (SE) response in various strains of chickens (Lamont et al., 2002) although some line broiler sires with specific alleles of the *NRAMP1* gene demonstrated enhanced resistance to the infection. Muhsinin et al. (2016) found that genetic heterogeneity in the resistance to *Sallmonell purllorum* in Indonesian native chickens could be linked to a polymorphism in *NRAMP1* in chickens. They also mentioned several SNP variations between local and foreign strains, as well as some amino acid changes, that could have a substantial impact on disease resistance in these chickens. In poultry, different SNPs have been linked to various traits. These findings suggest that SNPs have an important role in gene expression and, as a result, the expression of the protein or phenotype (Khoa et al., 2013; Aboukila et al., 2021).

The observed genetic similarities between indigenous chickens and hybrid broiler sequences may indicate the presence of similar capacities to resist pathogenic infections distinct from laying hens, suggesting that the presence of this gene and nucleotide variability may be implicated as major genetic factors in congenital diseases. Tohidi et al. (2013) and Liu et al. (2003) reported associations between SNP polymorphisms in *NRAMP1* and pathogen challenge responses in Leghorn chicks,

showing that either *NRAMP1* or related genes control the *Salmonella enteritidis* response traits.

In the present study, the sequencing of the *NRAMP1* gene in chickens was applied to two strains of chickens. In the *NRAMP1* gene, several SNPs were found in chickens. Desmond et al. (2019) discovered six SNPs in *NRAMP1* that were linked to amino acid changes in Nigerian and foreign chicken breeds. In this study, eight SNPs in *NRAMP1* were associated with amino acid changes.

CONCLUSION

Significant immunological differences between LB and Sinai strains, including albumin, IgY and HINDV, lymphocyte, and phagocyte activity, were discovered in this investigation. The alterations suggest that LB hens have a more evident cellular immune system as well as a general stronger innate and humoral immune response. On the other hand, Sinai (local chickens) exhibited a higher level of dominance in terms of Newcastle antibody titer. As a result, more consideration should be given to Sinai native chickens and the use of various crossing and selection procedures to generate egg-type native chicken lines to improve their productive features while preserving and benefiting from their remarkable Newcastle disease resistance. In addition, multiple SNPs in the *NRAMP1* gene have been discovered in Sinai, a native chicken, and LB, suggesting that this gene might be useful as a genetic marker for selecting high-producing indigenous hybrids with the ability to resist pathogenic diseases in poultry. In addition, substantial research is needed in various poultry species to examine the effect of polymorphisms on gene expression and the molecular mechanism induced by polymorphisms within this gene.

DECLARATIONS

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Availability of data and materials

Data will be available on request.

Authors' contributions

Walid Habashy (ORCID ID: 0000-0002-2009-5145) performed conceptualization and study design, methodology, formal analysis, and data curation. Walid Habashy and Manal Abdel-Rahman performed writing original draft preparation, Walid Habashy and Kwaku Adomako performed writing reviewing and editing. The manuscript has been read and approved by all authors.

Competing interests

The authors state that there is no conflict of interest with any financial, personal, or other relationships with other people or organizations that are relevant the material discussed in the manuscript.

Ethical consideration

The authors have examined ethical issues, such as consent to publish, misbehavior, data fabrication and/or falsification, duplicate publication and/or submission, and redundancy.

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