Livestock Science xxx (xxxx) xxx

Contents lists available at ScienceDirect

Livestock Science

journal homepage: www.elsevier.com/locate/livsci

Molecular associations of gallinacin genes with immune response against Salmonella typhimurium in chickens

Medhat S. Saleh [a], Maher H. Khalil [a,*], Mahmoud M. Iraqi [a], Antonio Camarda [b]

[a] Department of Animal Production, Faculty of Agriculture at Moshtohor, Benha University, 13736, Egypt
[b] Avian Pathology Section, Department of Veterinary Medicine, University of Bari, 70010 Valenzano - Bari, Italy

HIGHLIGHTS

- The *GAL 2* gene was homozygous, so it was excluded from the association analysis.
- The genotypes of *GAL 3, 4* and *5* genes were associated significantly with *S. typhimurium* count and antibody titer.
- The TT genotypes of *GAL 3* gene had higher significant *S. typhimurium* count and IgY antibody titer in R and ½R½F chickens.
- The AC genotype of *GAL 5* gene was the lowest significant for *S. typhimurium* count and IgA and IgY antibody titers in R and ½F½R chickens.

ARTICLE INFO

Keywords:
Fayoumi and Rhode Island Red chickens
Gallinacin genes
Polymorphic association
Salmonella
Antibody titers
Immune response

ABSTRACT

Candidate gallinacin genes (*GAL*) were assessed in Fayoumi (F), Rhode Island Red (R) and their crosses (½R½F and ½F½R) using PCR-RFLP technique to detect the associations between *GAL 2, GAL 3, GAL 4* and *GAL* 5 genes and caecal *S. typhimurium* bacterial count (CSTBC) and IgA, IgY and IgM antibody titers. The solutions of genotypes of *GAL* genes were calculated by the method of Generalized Least Squares (GLS). The SNPs genotypes of *GAL 3* and *GAL 5* genes showed significant counts of caecal *S. typhimurium*. The SNP of gallinacin *3*, 4 and 5 genes had significant effects on IgA, IgY and IgM antibody titers. For *GAL 3* gene, the chicks of genotype CC in R breed had lower significant CSTBC and higher significant IgA and IgM antibody titers than chicks of TT genotype, while the chicks of TC genotype had lower significant CSTBC in chicks of ½R½F crossbred and higher significant antibody titers of IgA and IgM in chicks of ½F½R crossbred. For *GAL 4* gene, the chicks of genotype GG in R breed had lower significant CSTBC and higher significant IgA, IgY and IgM antibody titers, but the chicks of genotype AG had higher significant IgA, IgY and IgM antibody titers in chicks of ½R½F crossbred than chicks of GG and AA genotypes. For *GAL 5* gene, the genotype CC in chicks of R breed had lower significant CSTBC and higher significant IgA and IgY antibody titers. In chicks of ½F½R crossbred, the chicks of genotype AA had lower significant CSTBC and higher significant IgA and IgY antibody titers than chicks of CA genotype. In practice, *GAL* genes could be used as markers assisted selection to improve immune response against *S. typhimurium* in genetic improvement programs of chickens.

1. Introduction

The advances in molecular technology have created a new horizon for the genetic improvement of disease-resistant traits in poultry. Several studies have exploited a priori knowledge of disease resistance and used the candidate gene approach for the identification of QTL in poultry. Detection of associations between candidate genes or markers and *Salmonella* bacterial burden could also lead to improve disease resistance in chickens (Ganz, 2003; Xiao et al., 2004; Muhsinin et al., 2017; Zhang et al., 2020; Ardiyana et al., 2020). The identification of direct or indirect molecular markers for these traits would facilitate the use of these markers in selection or in gene introgression (Wakchaure et al., 2015). The antimicrobial activity of avian ß–defensins have been identified by Higgs et al. (2005). Gallinacins 1 to 13 are functional analogues of the mammalian beta-defensins and play an important role in the innate immunity against bacterial infections in chickens (Ganz,

* Corresponding author.
E-mail addresses: medhat.saleh@fagr.bu.edu.eg (M.S. Saleh), maher.khalil@fagr.bu.edu.eg (M.H. Khalil), mahmoud.iraqi@fagr.bu.edu.eg (M.M. Iraqi), antonio.camarda@uniba.it (A. Camarda).

https://doi.org/10.1016/j.livsci.2020.104315
Received 8 August 2020; Received in revised form 27 October 2020; Accepted 27 October 2020
Available online 29 October 2020
1871-1413/

2003; Xiao et al., 2004; Hasenstein et al., 2006). However, *S. typhimurium* and *S. enteritidis* are acute systemic diseases in young chicks and few reports on *Salmonella* serovars distribution in broiler farms in Egypt were documented (Ammar et al., 2009; Abd El-Ghany et al., 2012).

Several researchers reported that there were significant associations between *NRAMP1, TGFβ3, TGFβ4, TLR4, TRAIL, GAL 4, GAL 5* and *GAL 14* candidate genes and immune traits against *Salmonella* in chickens (Tohidi et al., 2013; Muhsinin et al., 2016, 2017; Mamutse et al., 2018; Zhang et al., 2020). Tohidi et al. (2013) showed that CC genotype of *NRAMP1* gene was associated significantly with higher caecal *S. enteritidis* load. Muhsinin et al. (2017) showed that the genotype TT of *TGF-β2* gene was associated significantly with *S. pullorum* resistant in Sentul chickens. Mamutse et al. (2018) reported that GG genotype of *TLR4* gene had higher significantly immune response against Salmonella than AG and AA genotypes.

Extensive analysis of different inbred chickens has shown that some lines are consistently either susceptible or resistant to many serovars of *Salmonella* that have been tested, indicating a common resistance mechanism (Swaggerty et al., 2005; Fife et al., 2011), i.e. identifying susceptibility to *Salmonella* colonization in chickens and detecting the candidate genes that may contribute to disease resistance. Polymorphisms in *GAL 3, GAL 11, GAL 12* and *GAL 13* are associated with caecal bacterial load in chickens orally infected with *S. enteritidis* (Hasenstein and Lamont, 2007). Genetic variants in *TRAIL, TGFb3, CD28, MD-2, IL-10* and *MAPKAPK2* have been associated with caecal bacterial load (Malek and Lamont, 2003; Malek et al., 2004; Ghebremicael et al., 2008). The *TLR4* gene has been linked to resistance to infection with *S. typhimurium* in chickens (Leveque et al., 2003). Kramer et al. (2003) identified nine candidate genes namely *SLC11A1, IAP1, PSAP, CASP1, iNOS, IL2, IGL, TGFb2* and *TGFb4* that were associated with bacterial caecal load.

The current accessibility of the chicken genome sequence allied with high-density SNP panels provides an opportunity for a comprehensive analysis of *Salmonella* colonization QTL at a genome-wide level. This approach was reported early by Hasenstein et al. (2008) using two advanced intercross lines (AIL) to map QTL associated with host resistance to bacterial colonization. For five candidate gallinacin genes in poultry, Hasenstein et al. (2006) reported that *GAL 2* sire allele had a moderate association with progeny caecal bacterial load with no association with *S. enteritidis* antibody response, while *GAL 3* sire allele was associated with *S. enteritidis* antibody response and *GAL* 5 gene was moderately associated with antibody response to *S. enteritidis* vaccine. For studying the immune response in terms of gallinacin candidate genes located on chromosome 3 (*GAL 2, GAL 3, GAL 4* and *GAL 5*) and their associations with growth traits in chickens, Saleh et al. (2020) reported that *GAL 3, GAL 4* and *GAL 5* genes could be used in marker assisted selection programs to improve growth traits in chickens. However, investigations concerning associations of gallinacin genes with immune traits in chickens are scarce. In an attempt to investigate some of these concepts, Saleh et al. (2020) performed a crossbreeding experiment between Fayoumi (F) and Rhode Island Red (R) to estimate the crossbreeding effects in terms of direct, maternal and heterotic effects on body weights and gains and to detect the SNP associations of four immunity related gallinacin genes with body weights and gains in chickens. Here, the main objective of the present study was to detect the molecular associations between immune candidate gallinacin genes and their responses to *S. typhimurium* and antibody titers in chickens.

2. Materials and methods

2.1. Experimental animals

Fayoumi (F) and Rhode Island Red (R) and their crosses (½R½F and ½F½R) were used to detect polymorphic associations of gallinacin genes and immune traits against *S. typhimurium*. The details of breeding plan and management of the studied populations were described in our previous manuscript (Saleh et al., 2020). A total of 480 chicks were kept under similar hygienic and environmental conditions and provided un-medicated corn soybean-based meal diet (not containing antibiotics, coccidiostats, or growth promoters). The chicks were vaccinated in drinking water with the live attenuated virus vaccine of VMG91 103.0 Tissue Culture Infective Dose 50 for Infectious Bursal disease (Gumboro disease) at 14 and 21 days of age and with live lentogenic ND virus vaccine of LA SOTA 3.5 $\log_{10}$ Egg Infective Dose 50 for Newcastle disease at 18 and 28 days of age.

2.2. Caecal Salmonella typhimurium examined

The bacterial strain of *S. typhimurium* was obtained from Animal Health Research Institute of Agricultural Research Center, Giza, Egypt. The laboratorial examinations for bacterial count were carried out in the Labs of Research Park, Faculty of Agriculture, Benha University, Egypt. The media of nutrient broth and Salmonella and shigella (S.S) agar were used in identification and isolation of bacterial strain.

A total of 480 chicks were used and 120 chicks from each genetic group were infected with *S. typhimurium* at ten days of age (10^6 colony forming units (cfu) /chick). A total of 96 samples (24 from each genetic group) were collected from the caecum of chicks and examined for *S. typhimurium* presence at 10^{th} week of age using culture and quantification procedures described by Kaiser and Lamont (2001). At the beginning of the experiment, 15 chicks from each genetic group were randomly chosen and examined bacteriologically to ensure the absence of *Salmonella* from all chicks by cloacal swabs according to NMKL (1994).

Caecal material was serially diluted in sterile saline solution and plated on S.S agar. The plates incubated for 24 h at 37 °C, and colony forming units (cfu) were counted. The lowest number of *S. typhimurium* colonies that could be recovered by the plate count procedure was 100. If no colonies were recovered on the most concentrated dilutions of the plate count or by the enrichment procedure. At the 10^{th} week of age, 24 chicks from each genetic group were slaughtered and the caecal contents suspension was measured using thermo Orion pH meter after calibration with pH of 4.0, 7.0 and 10.0.

2.3. Examination of the antibody titers in the serum

The blood samples from 12 chicks of each genetic group were collected at the 4th week of age for ELISA test for measuring the antibody titers. The Calbiotech Inc. (CBI) *Salmonella* IgA, IgY, IgM ELISA Kits Cat#: ST093G (96 Tests) were used for the detection of IgA, IgY, IgM antibody titers to *Salmonella*. The collected blood specimens and separated serum and specimens were refrigerated at 2–8 °C for up to seven days or frozen for up to six months avoiding repetitive freezing and thawing. Prepared 1X wash buffer by adding the contents of the bottle (25 ml, 20X) to 475 ml of distilled or deionized water then stored at room temperature (18–26 °C). All specimens and kit reagents were brought to room temperature (18–26 °C) and gently mixed. The desired number of coated strips was placed into the holder. The 1:101 dilutions of test samples were prepared by adding 5 µl of the sample to 0.5 ml of the sample diluent and the mix of 100 µl of diluted sera, calibrator and controls were dispensed into the appropriate wells. For the reagent blank, 100 µl sample diluent in 1A well position was dispensed and tap the holder was used to remove air bubbles from the liquid, mixed well then incubated for 20 min at room temperature. Liquids were removed from all wells and washed three times with 300 µl of 1X wash buffer then blotted on absorbance paper or paper towel, then dispensed in 100 µl of enzyme conjugated to each well and incubated for 20 min at room temperature, The enzyme conjugated from all wells were removed and washed wells three times with 300 µl of 1X wash buffer then blotted on absorbance paper or paper towel, then dispensed in 100 µl of TMB substrate and incubated for 10 min at room temperature. A 100 µl of stop

solution was added and read Optical Density (O.D) at 450 nm using ELISA reader within 15 min. A dual wavelength was recommended with reference filter of 600–650 nm.

2.4. Blood sampling, DNA extraction and polymorphic assessment using PCR-RFLP

In the molecular genetic analyses, ninety-six blood samples belonging to four chicken genetic groups (24 samples from each group of F, R, ½R½F and ½F½R) were used. The laboratorial analyses for molecular biology were carried out in the Labs of Genetics Department, Faculty of Agriculture, Benha University, Egypt, and Avian Pathology Section, Department of Veterinary Medicine, University of Bari, Italy. Blood samples were collected from the wing vein by a 2-gage 1.5-injection needle into tubes containing EDTA. The genomic DNA extraction used Whole Blood Genomic DNA Purification Mini Kit (Cat No. #K0781, Thermo Scientific). The PCR primers, amplification and genotyping using PCR-RFLP technique of the same flock were described in our recent publication (Saleh et al., 2020).

2.5. Model for detecting the polymorphic associations between genotypes of gallinacin genes and studied traits

For detecting the associations between the genotypes of gallinacin genes and bacterial counts and immunity traits in each genetic group separately, the effects of genotypes of gallinacin genes SNPs were estimated using the PEST software (Groeneveld, 2006) and applying the following animal model:

$$\mathbf{y} = \mathbf{Xb} + \mathbf{Z_a u_a} + \mathbf{e}$$

Where y = the vector of observations of bacterial count or antibody titer trait; b = sex (males and females) and the genotypes of gallinacin gene (three genotypes for each SNP separately); X and Z_a= incidence matrices corresponding to fixed and additive random effects of the birds ($\mathbf{u_a}$), respectively; e = the residual error. The solutions of genotypes of *GAL* genes were calculated by the method of Generalized Least Squares (GLS) using the following equation:

$$\hat{\mathbf{b}} = \left(\mathbf{X}^{/}\mathbf{V}^{-}\mathbf{X}\right)^{-1}\mathbf{X}^{/}\mathbf{V}^{-}\mathbf{y}$$

Where X was the matrix of coefficients of estimable effects of gallinacin genes genotypes, $V-$ = the generalized error variance–covariance matrix, with the variance–covariance matrix of the estimate of b being: $\mathbf{Var\hat{b}} = (\mathbf{X}^{/}\boldsymbol{V}^{-}\boldsymbol{X})^{-1}$

3. Results and discussion

3.1. Molecular associations of gallinacin genes and studied traits

The generalized least square solutions of *S. typhimurium* count, caecal pH, and antibody titers detected in each genetic group for SNPs genotypes of *GAL* genes was varied (Tables 2, 3 and 4). Saleh et al. (2020) reported the *GAL 2* gene was one homozygous genotype in the four genetic groups, while in *GAL 3, GAL 4* and *GAL* 5 genes only one homozygous genotype in F breed was observed, so they were excluded from discussion of the association study. In general, the gene-trait associations that were identified in F_1 populations and robust in various genetic groups, and those identified SNPs are able to be widely used in marker-assisted selection.

3.2. Molecular associations of GAL 3 gene genotypes and studied traits

For gallinacin 3 gene, the counts of the *S. typhimurium* in the cecum are mostly significantly affected by SNP genotypes of *GAL* 3 gene (Table 1). The CC genotype in R breed had a lower *S. typhimurium* count of 2.0 than 3.12 cfu/g in TT genotype. But there was no significant difference in *S. typhimurium* count when compared to CC and TC genotypes. In ½R½F crossbred, the heterozygous TC genotype had a lower significant *S.* typhimuriumcount of 1.58 than that of 2.94 cfu/g in TT genotype and there was insignificant difference between TC and CC genotypes. There were non-significant differences in *S. typhimurium* count between TC and CC genotypes in ½F½R crossbred. Hasenstein and Lamont (2007) found that *GAL 3, GAL 11, GAL 12* and *GAL 13* genes had significant associations with cecum bacterial count in Broiler x Leghorn cross. With *NRAMP1* gene in Sentual chickens, Muhsinin et al. (2016) showed that CC genotype was significantly higher in immune resistance to *S. pullorum* than TC and TT genotypes ($p < 0.05$). In Egypt, Khatab et al. (2017) reported that chicks of F breed were conserved with one genotype (BB) for *TLR4*-exon 2 gene in disease resistance and susceptibility compared with Hy-line strain chickens, which have variable AB and BB genotypes. Zhang et al. (2020) reported that SNP1, SNP2, SNP12 and SNP17 of *GAL 14* gene were associated significantly with susceptibility of *Salmonella spp.*, and the other fifteen of *GAL 14* gene were not. Moreover, the genotypes TT of SNP1, TT of SNP2, GT of SNP12 and TT and AA of SNP17 were found to be susceptible for *Salmonella spp* and the genotypes CT and CC of SNP1, AT and AA of SNP2, GG and TT of SNP12 along with AT of SNP17 were found to be resistant to *Salmonella spp.*

Table 1
Generalized least square solutions (GLS) and their standard errors (SE) for the counts of *Salmonella typhimurium, ceacel* pH and antibody titers as affected by SNP genotypes of *GAL 3* gene in each genetic group separately.

Trait	Breed or genetic group[†]	Genotypes TT GLS	SE	TC GLS	SE	CC GLS	SE
S. typhimurium count (log cfu/g)	R	3.12^{a}	0.91	2.74^{ab}	0.91	2.0^{b}	0.41
	½R½F	2.94^{a}	0.82	1.58^{b}	0.47	1.77^{b}	0.68
	½F½R	–	–	1.76	0.47	1.0	0.32
Caecal *pH*	R	6.84	0.23	7.16	0.16	7.21	0.21
	½R½F	7.11	0.15	6.96	0.17	7.53	0.30
	½F½R	–	–	7.20^{a}	0.13	6.43^{b}	0.31
IgA antibody titer (OD)	R	0.75^{b}	0.30	0.70^{b}	0.30	1.25^{a}	0.35
	½R½F	1.29^{a}	0.35	1.22^{a}	0.17	0.81^{b}	0.50
	½F½R	–	–	1.09^{a}	0.34	0.76^{b}	0.24
IgY antibody titer (OD)	R	1.31^{a}	0.36	0.80^{b}	0.18	0.87^{b}	0.52
	½R½F	1.38^{a}	0.39	1.12^{ab}	0.22	0.91^{b}	0.19
	½F½R	–	–	1.02	0.35	0.86	0.24
IgM antibody titer (OD)	R	0.79^{b}	0.27	0.80^{b}	0.20	1.28^{a}	0.27
	½R½F	1.33^{a}	0.40	1.09^{ab}	0.20	0.84^{b}	0.56
	½F½R	–	–	1.01^{a}	0.36	0.73^{b}	0.26

[†] R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; GLS= generalized least square solutions; SE= standard errors; Different letters in the same row indicate significant differences at $p < 0.05$; cfu= colony forming units; OD= optical density.

For caecal pH, there were non-significant differences in caecal pH among the genotypes of R and ½R½F chickens, while in ½F½R crossbred the TC genotype had significant higher pH value of 7.20 than that of 6.43 in CC genotype (Table 1).

The SNP of *GAL 3* gene had significant effects on IgA, IgY and IgM antibody titers (Table 1). The genotype CC in R breed had high significant IgA antibody titers of 1.25 OD than 0.70 OD in TC genotype and 0.75 OD in TT genotype while, there was insignificant association between TT and TC genotypes. Similarly, the genotype CC had high significant IgM antibody titers of 1.28 OD than 0.80 OD in TC genotype and 0.79 OD in TT genotype and there were significant associations between TT and TC genotypes. The genotype TT had higher significant IgY antibody titer of 1.31 OD than 0.80 and 0.87 OD in TC and CC genotypes, respectively. The homozygous genotype TT in chicks of ½R½F crossbred had higher significant antibody titers of 1.29 OD for IgA than 0.81 OD for CC genotype and there were insignificant differences

between TT and TC genotypes. The genotype TT in chicks of ½R½F crossbred had higher significant IgY antibody titers of 1.38 OD than 0.91 OD for CC genotype. The chicks of genotype TT in ½R½F crossbred had higher significant IgM antibody titers of 1.33 OD than 0.84 OD for CC genotype. In chicks of ½F½R crossbred, the genotype TC had higher significant antibody titers of 1.09 and 1.01 OD than 0.76 and 0.73 OD in CC genotype for IgA and IgM, respectively. Hasenstein et al. (2006) found that *GAL 3* gene was associated significantly with *S. enteritidis* antibody response in F_1 chicks ($p < 0.03$).

3.3. Molecular associations among genotypes of GAL 4 gene and studied traits

The generalized least square solutions for SNP genotypes of *GAL 4* gene showed that the genotypes GG and AA in chicks of R breed had lower significant *S. typhimurium* count of 1.83 and 1.89 cfu/g than that of 3.0 cfu/g for AG genotype (Table 2). In chicks of ½R½F and ½F½R crossbreds the differences among genotypes were non-significant, but the genotype GG had a lower *S. typhimurium* than other genotypes. Hasenstein et al. (2006) found that *GAL 4* gene had insignificant association ($p < 0.24$) with caecal *S. enteritidis* count in F_1 generation. In chicks of intercross line, Hasenstein and Lamont (2007) showed that *GAL 1, GAL 2, GAL 4, GAL 7, GAL 8, GAL 9* and *GAL 10* genes were associated insignificantly with caecal *Salmonella* bacterial count. Zhang et al. (2020) stated that the genotypes CT, TG and GG of SNP1, SNP2 and SNP12 of *GAL 4* gene were associated insignificantly with susceptibility to *Salmonella spp*.

The differences in caecal pH among the three genotypes in chicks of R, ½R½F and ½F½R genetic groups were non-significant (Table 2).

The SNP of gallinacin 4 gene had significant effects on IgA, IgY and IgM antibody titers (Table 2). The genotype GG in chicks of R breed had higher significant IgA antibody titers of 1.42 OD than 1.0 OD in AG genotype and 0.91 OD in AA genotype and there were insignificant associations between AA and GG genotypes. Similarly, the chicks of genotype GG in R breed had higher significant IgY antibody titers of 1.44 OD than 1.0 OD in AG genotype and 0.93 OD in AA genotype while, there were insignificant differences between AA and GG genotypes. The chicks of homozygous genotype GG in R breed had higher significant IgM antibody titers of 1.48 OD than 1.0 OD in AG genotype and 0.90 OD in AA genotype and the significant differences were not detected between AA and GG genotypes. In chicks of ½R½F, the genotype AG had higher significant IgA antibody titers of 1.37 OD than 0.82 OD in GG genotype and 0.88 OD in AA genotype and there were insignificant associations between AA and GG genotypes. The genotype AG in chicks of ½R½F crossbred had higher significant IgY antibody titers of 1.41 OD than 0.92 OD in GG genotype and 0.87 OD in AA genotype while, there were insignificant differences between AA and GG genotypes. Also, the chicks of genotype AG in ½R½F crossbred had higher significant IgM antibody titers of 1.32 OD than 0.89 OD in GG genotype and 0.87 OD in AA genotype and the significant differences were not detected between AA and GG genotypes. In chicks of ½F½R crossbred, the differences between the three genotypes were non-significant. Hasenstein et al. (2006) showed that there was insignificant association between SNP of *GAL 4* gene and antibody responses against *S. enteritidis* ($p < 0.79$).

3.4. Molecular associations among genotypes of GAL 5 gene and studied traits

The generalized least square solutions for SNP genotypes of *GAL 5* gene showed counts of caecal *S. typhimurium* are significant (Table 3). In R breed, the AC and CC genotypes had a lower significant *S. typhimurium* count of 2.0 and 2.25 cfu/g than 3.04 cfu/g for AA genotype. In ½R½F crossbred, there were non-significant differences between CC and CA genotypes for *S. typhimurium*. The genotypes AA and AC in ½F½R crossbred had a lower significant *S. typhimurium* count of 1.0 and 1.13 cfu/g than 2.11 cfu/g for CC genotype. Hasenstein et al. (2006) reported insignificant association ($p < 0.45$) between *GAL 5* SNP and caecal *Salmonella* count. Zhang et al. (2020) reported that five SNPs (SNP2, SNP10, SNP15, SNP16 and SNP17) of *GAL 5* gene had significant associations with susceptibility to *Salmonella spp.,* and the other fifteen SNPs were not. Moreover, the genotypes AG of SNP2, AA of SNP10, CC of SNP15, CC of SNP16 and TT of SNP17 were found to be susceptible to *Salmonella spp.,* while the genotypes AA of SNP2, AG and GG of SNP10, TC and TT of SNP15, TC and TT of SNP16 along with TC and CC of SNP17 were found to be resistant to *Salmonella spp*.

The differences in caecal pH between the other genotypes in R, ½R½F

Table 2
Generalized least square solutions (GLS) and their standard errors (SE) for the counts of *Salmonella typhimurium*, ceacel pH and antibody titers as affected by SNP genotypes of *GAL 4* gene in each genetic group separately.

Trait	Breed or genetic group[†]	Genotypes AA		AG		GG	
		GLS	SE	GLS	SE	GLS	SE
S. typhimurium count (log cfu/g)	R	1.89[b]	0.83	3.0[a]	0.61	1.83[b]	0.58
	½R½F	1.73	0.41	1.99	0.63	2.11	0.51
	½F½R	–	–	1.62	0.35	1.65	0.59
Caecal *pH*	R	7.17	0.16	7.11	0.16	7.21	0.39
	½R½F	7.01	0.16	7.23	0.19	7.08	0.32
	½F½R	–	–	7.28	0.25	7.08	0.14
IgA antibody titer (OD)	R	0.91[b]	0.60	1.0[b]	0.18	1.42[a]	0.17
	½R½F	0.88[b]	0.39	1.37[a]	0.19	0.82[b]	0.24
	½F½R	–	–	0.91	0.42	0.94	0.22
IgY antibody titer (OD)	R	0.93[b]	0.59	1.0[b]	0.18	1.44[a]	0.17
	½R½F	0.87[b]	0.24	1.41[a]	0.40	0.92[b]	0.20
	½F½R	–	–	0.83	0.43	0.94	0.23
IgM antibody titer (OD)	R	0.90[b]	0.40	1.0[b]	0.29	1.48[a]	0.47
	½R½F	0.87[b]	0.27	1.32[a]	0.39	0.89[b]	0.22
	½F½R	–	–	0.80	0.45	0.92	0.24

[†] R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; GLS= generalized least square solutions; SE= standard errors; Different Letters in the same row indicate significant differences at $p<0.05$; cfu= colony forming units; OD= optical density.

Table 3
Generalized least square solutions (GLS) and their standard errors (SE) for the counts of *Salmonella typhimurium*, ceacel pH and antibody titers as affected by SNP genotypes of *GAL 5* gene in each separate genetic group.

Trait	Breed or genetic group[†]	Genotypes AA		AC		CC	
		GLS	SE	GLS	SE	GLS	SE
S. typhimurium count (log cfu/g)	R	3.04[a]	0.63	2.0[b]	0.54	2.25[b]	0.61
	½R½F	–	–	1.94	0.64	1.58	0.33
	½F½R	1.0[b]	0.28	1.13[b]	0.28	2.11[a]	0.59
Caecal *pH*	R	7.44	0.36	7.09	0.52	7.60	0.13
	½R½F	–	–	7.13	0.24	7.0	0.12
	½F½R	7.50	0.39	7.09	0.13	7.40	0.27
IgA antibody titer (OD)	R	1.51[a]	0.62	0.77[b]	0.16	1.38[a]	0.34
	½R½F	–	–	1.22	0.15	0.89	0.29
	½F½R	1.38[a]	0.15	0.90[b]	0.31	1.34[a]	0.45
IgY antibody titer (OD)	R	1.57[a]	0.16	0.85[b]	0.43	1.41[a]	0.31
	½R½F	–	–	1.46	0.30	1.03	0.15
	½F½R	1.30[a]	0.15	0.84[b]	0.44	1.32[a]	0.31
IgM antibody titer (OD)	R	0.87[c]	0.32	1.67[a]	0.61	1.25[b]	0.53
	½R½F	–	–	1.36	0.26	1.04	0.13
	½F½R	1.37	0.17	1.02	0.32	1.31	0.45

[†] R= Rhode Island Red breed; ½R½F = Rhode Island Red × Fayoumi; ½F½R= Fayoumi × Rhode Island Red; GLS= generalized least square solutions; SE= standard errors; Different letters in the same row indicate significant differences at $p<0.05$; cfu= colony forming units; OD= optical density.

and ½F½R genetic groups were non-significant (Table 3).

The SNP of gallinacin 5 gene had significant effects on IgA, IgY and IgM antibody titers (Table 3). The genotypes AA and CC in R breed had higher significant values of 1.51 and 1.38 OD for IgA antibody titer and 1.57 and 1.41 OD for IgY antibody titer than the respective antibody titer of 0.85 OD in CA genotype, while the genotype CA had a higher significant IgM antibody titer of 1.67 than 1.25 and 0.87 OD in CC and AA genotypes, respectively. In ½F½R breed, the genotypes AA and CC had higher significant values of 1.38 and 1.34 OD for IgA antibody titer than 0.90 OD in CA genotype and the values of 1.30 and 1.32 OD for IgY antibody titer in AA and CC genotypes than the corresponding antibody titers of 0.84 OD in CA genotype, while the differences in antibody titers between the three genotypes in ½R½F crossbred were non-significant. Hasenstein et al. (2006) reported that gallinacin 5 gene showed moderate associations between *GAL 5* SNP and antibody responses against *S. enteritidis* ($p < 0.11$).

The chickens' immune system composed of both innate and acquired immunity. The adaptive immune system eliminates the pathogens in two ways: one through the production of immunoglobulin by B-cells, referred to as humeral immune response, which operates by means of specific antibodies (Kean et al., 1994; Cheema et al., 2003; Barrow, 2007; Tohidi et al., 2018). Several studies have been reported an increase in antibody levels, primarily immunoglobulin IgY and IgA (Beal et al., 2004; Barrow, 2007; Barrow et al., 2012). Although antibodies are known to be important in controlling *Salmonella* infection, their exact role remains unclear (Restif et al., al., 2013; Dar et al., 2019). Recent studies have rekindled our interest to unveil the role of serum antibodies against *Samonella typhimurium*. Strong antigen-specific cell and humeral immune responses have both been temporally linked to clearance of *Samonella typhimurium* infection in chicks (Saif et al., al., 2008; Beal and smith, 2007; Dar et al., 2019). In practice, Adaptive immunity is required to specifically focus defense mechanisms on that particular pathogen resulting not only in the elimination of the pathogen but also as protection in case of a repeat encounter with the same pathogen (Brisbin et al., 2008; Iwasaki and Medzhitov, 2015; Swaggerty et al., 2019). It is the ability of adaptive immunity to recognize molecular features of the pathogen using highly specific antigen receptor-antigen interactions that conveys specificity to adaptive immunity and allows it to specifically focus immune activities on the invading pathogen. Therefore, determining the genetic bases of these immunological parameters IgA, IgY and IgM antibody titers against *S. typhimurium* are of considerable interest, as this information could be used to select for chicks with superior adaptive immune response.

4. Conclusions

The counts of caecal *S. typhimurium* along with antibody titers are greatly affected by SNP genotypes of gallinacin 3, 4 and 5 genes in poultry. Therefore, the *GAL 3, GAL 4* and *GAL 5* genes could be used as genetic markers in selection programs to improve the genetic immune response against *S. typhimurium* in chickens. Also, it is possible to use *GAL* genes in poultry breeding programs in order to reduce significantly the amounts and costs of drugs and to prevent the decline in production performance. Due to the limited sample size some associations in this study could be less reliable. Thus, larger sample size is needed for further validation.

Availability of data and materials

The data used in the present study were obtained from the experiment performed in the Poultry Farm, Faculty of Agriculture, Benha University, Egypt. Data used and analyzed are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All experimental procedures were approved by Animal Production Department, Faculty of Agriculture, Benha University, Egypt (Approval Number: 2016–1).

CRediT authorship contribution statement

Medhat S. Saleh: Conceptualization, Data curation, Formal analysis, Software, Writing - original draft. **Maher H. Khalil:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing - review & editing. **Mahmoud M. Iraqi:** Conceptualization, Methodology, Supervision, Validation, Visualization, Writing - review & editing. **Antonio Camarda:** Conceptualization, Data curation, Formal analysis, Supervision, Writing - review & editing.

Declaration of Competing Interest

The authors declare that there is no conflict of interest for this study.

Acknowledgments

The authors would like to thank the European Union Commission that partially funded this Joint Master Degree of TEMPUS program (Project No. 543865). The authors are gratefully acknowledged the support and help of members of Research Labs Park and Labs of Genetics Department, Faculty of Agriculture, Benha University, Egypt, and Molecular Biology Labs of Avian Pathology Section, Department of Veterinary Medicine, University of Bari, Italy.

Funding

This work was supported and funded by Faculty of Agriculture at Moshtohor, Benha University, Egypt and Tempus European Union program, Project No. 543865.

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.livsci.2020.104315.

References

Abd El-Ghany, W.A., Abd El-Shafii, S.S., Hatem, M., 2012. A survey on Salmonella species isolated from chicken flocks in Egypt. Asian J. Anim. Vet. Adv. 7, 489–501.

Ammar, A., Ahmed, Y., Asawy, A., Ibrahim, A., 2009. Bacteriological studies on Salmonella enteritidis isolated from different sources in Dakhlia Governorate. Assiut Vet. Med. J. 56, 125–135.

Ardiyana, M., Gunawan, A., Murtini, S., Sartika, T., Sumantri, C., 2020. Polymorphisms and associations of the NRAMP-1 and iNOS genes on Newcastle disease and Salmonella enteritidis resistances in SenSi-1 Agrinak Chickens. Trop. Anim. Sci. J. 43, 95–102.

Barrow, P.A., 2007. Salmonella infections: immune and non-immune protection with vaccines. Avian. Pathol. 36 (1), 1–13. https://doi.org/10.1080/03079450601113167.

Barrow, P.A., Jones, M.A., Smith, A.L., Wigley, P., 2012. The long view: salmonella–the last forty years. Avian. Pathol. 41 (5), 413–420. https://doi.org/10.1080/03079457.2012.718071.

Beal, R.K., Smith, A.L., 2007. Antibody response to Salmonella: its induction and role in protection against avian enteric salmonellosis. Expert Rev. Anti Infect. Ther. 5 (5), 873–881. https://doi.org/10.1586/14787210.5.5.873. PMID: 17914920.

Beal, R.K., Powers, C., Wigley, P., Barrow, P.A., Smith, A.L., 2004. Temporal dynamics of the cellular, humoral and cytokine responses in chickens during primary and secondary infection with Salmonella enterica serovar Typhimurium. Avian. Pathol. 33 (1), 25–33. https://doi.org/10.1080/03079450310001636282. PMID: 14681065.

Brisbin, J.T., Gong, J., Sharif, S., 2008. Interactions between commensal bacteria and the gut-associated immune system of the chicken. Anim. Health Res. Rev. 9 (1), 101.

Cheema, M.A., Qureshi, M.A., Havenstein, G.B., 2003. A comparison of the immune response of a 2001 commercial broiler with a 1957 random bred broiler strain when fed representative 1957 and 2001 broiler diets. Poult. Sci. 82, 1519–1529.

Dar, M.A., Urwat, U., Ahmad, S.M., Ahmad, R., Kashoo, Z.A., Dar, T.A., Bhat, S.A., Mumtaz, P.T., Shabir, N., Shah, R.A., Heidari, M., 2019. Gene expression and

antibody response in chicken against Salmonella Typhimurium challenge. Poult. Sci. 98 (5), 2008–2013.
Fife, M., Howell, J., Salmon, N., Hocking, P., Van Diemen, P., Jones, M., Stevens, M., Kaiser, P., 2011. Genome-wide SNP analysis identifies major QTL for Salmonella colonization in the chicken. Anim. Genet. 42, 134–140.
Ganz, T., 2003. Defensins: antimicrobial peptides of innate immunity. Nat. Rev. Immunol. 3, 710–720.
Ghebremicael, S.B., Hasenstein, J.R., Lamont, S.J., 2008. Association of interleukin 10 cluster genes and Salmonella response in the chicken. Poult. Sci. 87, 22–26.
Groeneveld, E., 2006. PEST User's Manual. Institute of Animal Husbandry and Animal Behaviour, FAL, Germany.
Hasenstein, J.R., Lamont, S.J., 2007. Chicken gallinacin gene cluster associated with Salmonella response in advanced intercross line. Avian. Dis. 51, 561–567.
Hasenstein, J.R., Zhang, G., Lamont, S.J., 2006. Analyses of five gallinacin genes and the Salmonella enterica serovar enteritidis response in poultry. Infect. Immun. 74, 3375–3380.
Hasenstein, J.R., Hassen, A.T., Dekkers, J.C., Lamont, S.J., 2008. High resolution, advanced intercross mapping of host resistance to Salmonella colonization. Dev. Biol. 132, 213–218. https://doi.org/10.1159/000317162.
Higgs, R., Lynn, D.J., Gaines, S., Mcmahon, J., Tierney, J., James, T., Lloyd, A.T., Mulcahy, G., O'farrelly, C., 2005. The synthetic form of a novel chicken β-defensin identified in silico is predominantly active against intestinal pathogens. Immunogenetics 57, 90–98.
Iwasaki, A., Medzhitov, R., 2015. Control of adaptive immunity by the innate immune system. Nat. Immunol. 16, 343–353. https://doi.org/10.1038/ni.3123.
Kaiser, M., Lamont, S.J., 2001. Genetic line differences in survival and pathogen load in young layer chicks after Salmonella enterica serovar Enteritidis exposure. Poult. Sci. 80, 1105–1108.
Kean, R.P., Cahaner, A., Freeman, A.E., Lamont, S.J., 1994. Direct and correlated responses to multitrait, divergent selection for immunocompetence. Poult. Sci. 73, 18–32.
Khatab, S.A., Hemeda, S.A., El-Nahas, A.F., Abd El Naby, W.S., 2017. Polymorphisms of TLR4 gene and its association with genetic resistance to Salmonella Enteritidis infection in Fayoumi breed and Hy-line strain in Egypt. Alex. J. Vet. Sci. 55, 1–9.
Kramer, J., Malek, M., Lamont, S.J., 2003. Association of twelve candidate gene polymorphisms and response to challenge with Salmonella enteritidis in poultry. Anim. Genet. 34, 339–348.
Leveque, G., Forgetta, V., Morroll, S., Smith, A.L., Bumstead, N., Barrow, P., Loredo-Osti, J.C., Morgan, K., Malo, D., 2003. Allelic variation in TLR4 is linked to susceptibility to Salmonella enterica serovar Typhimurium infection in chickens. Infect. Immun. 71, 1116–1124.
Malek, M., Lamont, S.J., 2003. Association of INOS, TRAIL, TGF-β2, TGF-β3, and IgL genes with response to Salmonella enteritidis in poultry. Gen. Sel. Evol. 35, 99–111.
Malek, M., Hasenstein, J.R., Lamont, S.J., 2004. Analysis of chicken TLR4, CD28, MIF, MD-2, and LITAF genes in a Salmonella enteritidis resource population. Poult. Sci. 83, 544–549.
Mamutse, J., Gunawan, A., Sumantri, C., Murtini, S., Sartika, T., 2018. Association of the Toll-like Receptor 4 (TLR4) and Myxovirus (Mx) genes with resistance to Salmonella and Newcastle disease in selected Sentul chickens. Poult. Sci. 17, 591–599.
Muhsinin, M., Ulupi, N., Gunawan, A., Wibawan, I.W.T., Sumantri, C., 2016. Association of NRAMP1 polymorphisms with immune traits in Indonesian native chickens. Int. J. Poult. Sci. 15, 401–406.
Muhsinin, M., Ulupi, N., Gunawan, A., Wibawan, I.W.T., Sumantri, C., 2017. g. 640T>C polymorphism of the TGF-β2 gene is associated with Salmonella pullorum resistance in Indonesian chickens. Anim. Prod. Sci. 19, 81–92.
NMKL, 1994. Report No. 5, 2nd Ed..
Restif, O., Goh, Y.S., Palayret, M., Grant, A.J., McKinley, T.J., Clark, M.R., Mastroeni, P., 2013. Quantification of the effects of antibodies on the extra-and intracellular dynamics of Salmonella enterica. J. R. Soc. Interface 10 (79), 20120866.
32 Saif, Y.M., Barnes, H., Glisson, J.R., Fadly, A.M., McDougald, L.R., Swayne, D.E., 2008. Diseases of poultry. 12. Blackwell Pub Professional, Ames, Iowa, pp. 452–514.
Saleh, M.S., Iraqi, M.M., Khalil, M.H., Camarda, A., 2020. Crossbreeding analyses and polymorphic associations of gallinacin genes with growth traits in chickens. Livest. Sci. 240 (2020), 104118 https://doi.org/10.1016/j.livsci.2020.104118i.
Swaggerty, C.L., Ferro, P.J., Pevzner, I.Y., Kogut, M.H., 2005. Heterophils are associated with resistance to systemic Salmonella enteritidis infections in genetically distinct chicken lines. FEMS Immunol. Med. Mic. 43, 149–154.
Swaggerty, C.L., Callaway, T.R., Kogut, M.H., Piva, A., Grilli, E., 2019. Modulation of the immune response to improve health and reduce foodborne pathogens in poultry. Microorganisms 7 (3), 65. https://doi.org/10.3390/microorganisms7030065.
Tohidi, R., Idris, I., Javanmard, A., 2018. Immunogenetics applied to control salmonellosis in chicken: a review. J. Appl. Anim. Res. 46, 331–339. https://doi.org/10.1080/09712119.2017.1301256.
Tohidi, R., Idris, I., Malar Panandam, J., Hair Bejo, M., 2013. The effects of polymorphisms in 7 candidate genes on resistance to Salmonella Enteritidis in native chickens. Poult. Sci. 92, 900–909.
Wakchaure, R., Ganguly, S., Praveen, P., Kumar, A., Sharma, S., Mahajan, T., 2015. Marker assisted selection (MAS) in animal breeding: a review. J. Drug Metab. Toxicol. 6, 127–130.
Xiao, Y., Hughes, A.L., Ando, J., Matsuda, Y., Cheng, J.-F., Skinner-Noble, D., Zhang, G., 2004. A genome-wide screen identifies a single β-defensin gene cluster in the chicken: implications for the origin and evolution of mammalian defensins. BMC Genomics 5, 56–66.
Zhang, L., Huang, M., Li, Y., Chen, D., Shi, X., 2020. Association of three beta-defensin gene (AvBD4, AvBD5, AvBD14) polymorphisms with carrier-state susceptibility to salmonella in chickens. Br. Poult. Sci. https://doi.org/10.1080/00071668.2020.1752913, 2020 May:1-9.