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Estimating genetic parameters of immunocompetent traits in Rhode Island Red chicken#

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ABSTRACT

The study aimed to estimate genetic parameters of immunocompetent traits in Rhode Island Red (RIR) chicken. Five to six weeks aged pedigreed RIR chicks of a selected (79 chicks) and a control (74 chicks) line maintained at the Central Avian Research Institute were immunized against 1% (v/v) SRBC suspension. Sera collected on 5th day post immunization were analyzed through haemagglutination (HA) test to assess humoral immune response. Serum lysozyme and immunoglobulin-G (IgG) concentrations were assessed through agarose lysoplate and single radial immunodiffusion assay. The data were analyzed by least squares analysis of variance. The least squares mean estimates of serum lysozyme and IgG were significantly influenced by sires in the selected line, but any estimate was not affected by sex in any line. The selected line had more serum lysozyme than the control line, which contained more HA titre and serum IgG. The traits were heritable at a range of 0.105 to 0.398, and correlated with a low to high range (0.134 to 0.552) of genetic correlations and least to low range (0.007 to 0.170) of phenotypic correlations. The information generated could be important while selecting birds for improvement in general immune responsiveness.

Key words: Correlations, HA titre, Heritability, IgG, Immunocompetence, RIR chicken lines, Serum lysozyme

Since the inception of the Central Avian Research Institute at Izatnagar (India) in 1979, this institute has been maintained Rhode Island Red (RIR) chicken germplasm brought from the USA. The germplasm was subjected to genetic selection. The population was acclimatized and genetically improved over last 33 years covering 29 generations of selection and being maintained as selected line. A random bred control population is being maintained since then. Genetic variability and relatedness among these chicken lines are prerequisite information required for because the genetic variation is considered as the primary biological resource that can be exploited in breeding programmes (Das *et al.* 2014a). Understanding of the genetics of various immunological traits involved in immunocompetence provides significant information to be used in programmes for genetic improvement of birds with higher production and protection status. Van der Zijpp *et al.* (1983) suggested that genetic parameters should be combined while selecting for improvement in general immune responsiveness, owing to low and non-significant correlations among immunocompetence traits. One of the important non-pathogenic, multi-determinant antigens to monitor immune responsiveness in poultry is sheep red blood cells (SRBC) (Siegel and Gross 1980). Birds eliciting higher antibody response against SRBC also produce more antibodies to a variety of antigens (Parmentier *et al.* 1998). The non-specific components of immune system like lysozyme play an important role in the body's defense against infection through stimulatory effect on phagocytic function of macrophages as well as its direct bacteriolytic action (Das *et al.* 2014b). Lysozyme exists in high concentration in egg-white and developing embryo of many birds, also present in various body fluids such as saliva, sera, tears, tracheobrachial secretion, gastric juice etc. and acts as first line of defense to protect the animal being get infected by bacteria. The lysozyme has the ability to break down the peptidoglycan layer in the cell wall of Gram negative bacteria and thus kills the bacteria. The IgG is the most abundant immunoglobulin in serum and regarded as an indicator of general immune response (Pinard Van der Laan *et al.* 1998). The bird's ability to mount antibody

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responses to other antigen is primarily revealed by serum IgG concentration which is traceable in all body fluids. The present investigation was carried out to estimate genetic parameters of humoral immune response to SRBC (HA titre), serum lysozyme and immunoglobulin-G concentrations through estimating their least squares means, least squares analysis for sire, sex and genotype component of variances, and estimating heritability and correlation parameters from sire component of variance in the selected and control line of Rhode Island Red chicken.

MATERIALS AND METHODS

Experimental chicks and husbandry adopted: A total of 153 number of 5 to 6 wk aged single hatched out pedigreed chicks of the selected (79 progenies of 28 sires) and control (74 progenies of 16 sires) line of RIR chicken maintained at experimental layer farm of this institute were investigated. The day-old chicks were wing banded and pedigreed by sire and dam in the hatchery itself along with following a standard vaccination schedule being followed at this institute (Das *et al.* 2014a, Das *et al.* 2014b). Standard litter brooding, housing and *ad libitum* feeding were provided with optimum management (Das *et al.* 2014d).

Immunization of the chicks and harvesting of immune sera: The chicks (5–6 weeks aged) were immunized against sterile SRBC suspension *de novo* prepared from the venous blood collected from Muzaffarnagari sheep maintained at the sheep and goat farm. The hyper immune sera were harvested in 0.5 ml sterile tubes from approximate 1 ml of anticoagulant-free blood collected on 5^{th} day post immunization (5 dpi) from jugular vein or wing vein of the chicks immunized with 1 ml of 1% (v/v) sterile SRBC suspension in phosphate buffer saline (PBS, pH 7.4). For this, the blood collected in 1.5 ml sized sterile tubes was allowed to clot keeping the tubes slant way on sun ray. The sera samples were stored at –20ºC till further analysis.

Estimation of immunological traits: The humoral immune response of the immunized chicks was assessed by estimating *in vivo* antibody response to SRBC through haemagglutination (HA) test (Van der Zijpp and Leenstra 1980) with a principle of interaction between antibody and a particular antigen results in visible clumping called agglutination. The test was performed in U shaped round bottom micro-titre plates. PBS (50 ml) was added in each well and 50 ml of serum was added in first well of each row except the last row where 50 ml of PBS was added, the last row would act as control. After thorough mixing, the sera was 2-fold serially diluted by taking 50 ml from first wells of each row and transferring it into the subsequent wells. This process was continued till last column from where 50 ml was discarded. Equal volume (50 ml) of 1% (v/v) SRBC suspension was then added in all the wells followed by thorough mixing by rocking plates on table surface. The plates were then incubated at 37ºC for 1 h in a humid chamber. The highest dilution (n) of sera that yielded complete agglutination was recorded as titre and then expressed as $\log_2 n$ (Siegel and Gross 1980).

The serum lysozyme concentration was estimated through lysoplate assay (Lie *et al.* 1986) using 1% agarose as solidifying base onto a clean and sterilized glass plate placed on smooth leveled horizontal surface. The borders of the gel were prepared by placing glass strips on the edges of required area. All the four sides of the borders were sealed with 2% agar. Volume for 0.5 cm thick gel (in ml) was calculated as length × width × 0.5 cm. After boiling, 1% agarose in the required dibasic buffer was allowed to cool to 60ºC and pre-diluted gram negative *Micrococcus lysodieketicus* bacterium @ 50 mg / ml of the dibasic buffer One thousand ml of dibasic buffer (0.066 M, pH 6.3) contains 100 ml Na_2HPO_4 (1M), 100 ml $Na_2H_2PO_4$ (1M) and 800 ml triple distilled water. required for the gel was added to it. Dibasic buffer is used as a buffering agent for preparation of the agarose solution where the *M. lysodieketicus* bacterium can grow. After thorough mixing, the whole content of the boiled and cooled agarose solution was poured onto the glass plate, and allowed to spread uniformly. It was left at room temperature for 45 min so that get the gel gets polymerized. The wells were punched at a distance of approximately 1.75 cm with the help of a gel puncher (5.4 mm diameter). Lysozyme stock solution was prepared diluting 2 µg standard lysozyme into 1 µl dibasic buffer. Two-fold serial dilutions of the stock solution were made to get the final concentrations of standard lysozyme as 40 µg/ml, 20 µg/ml, 10 µg/ml, 5.0 µg/ml, 2.5 µg/ml and 1.25 µg/ml as working solutions. These working lysozyme standards were loaded in the wells in a row on to the lysoplate in order to plot standard curve. Then 10 µl of each unknown sera sample was loaded in the rest wells onto the lysoplate.

The serum IgG concentrations were assessed through single radial immunodiffusion (SRID) assay (Mancini *et al.* 1965) using 3% (w/v) agarose in 0.1M Tris-HCl as solidifying base onto a clean and sterilized glass plate as placed, structured and volume-determined as for lysoplate. Total volume of 0.1 M Tris-HCl required for the gel preparation was divided equally into two halves. To the first half, total amount of agarose required was added and boiled to dissolve the agarose. To the second half, total amount of rabbit anti-chicken IgG @ 35 µl/ml of 0.1M Tris-HCl required for the gel was added. Chicken serum IgG neutralizes the rabbit anti-chicken IgG. After boiling, the first half with agarose was kept at 50ºC in a water bath. The temperature of the first half was brought down to about 50ºC and the second half was mixed. The whole content was then poured onto the glass plate. The gel was allowed to solidify at room temperature for around 45min; wells were punched at a distance of 1.5 cm with the help of gel puncher (2.5 mm diameter). The standard chicken IgG (IgY) stock solution was prepared diluting 25 mg IgY into 1 ml 0.1M Tris-HCl. Working standards of 25 mg/ml, 12.5 mg/ml, 6.25 mg/ml, 3.125 mg/ml and 1.562 mg/ml concentrations were prepared by serial dilution of the IgY stock solution and loaded in the wells in a row to plot standard curve. Unknown sera (5 µl) was diluted to 4 times with 0.1M Tris-HCl and 10 ul of each sera sample was loaded in the rest wells.

The lysoplate and IgG plate were incubated at 37ºC in humidity-controlled chamber for 24 h, thereafter stained with 0.2% coomassie brilliant blue staining solution for 6 h and excess stain was removed with destaining solution. In lysoplate assay, *M. lysodiekticus* bacterium is distributed evenly in the agarose plate. The serum lysozyme when loaded into the wells in the agarose plate moves radialy outwards and kill the bacterium. A ring is formed around the well which shows complete lysis of the bacterium. The diameter of ring is proportional to the concentration of lysozyme. In SRID assay, the serum antibody (IgG) when loaded into the wells diffuses into the agarose containing suitable dilution of an antiserum (rabbit anti-chicken IgG), the region of equivalence is established and a ring of precipitation is formed around the well. The area of precipitation ring is proportional to concentration of serum IgG loaded. The diameters of the lysed zones around standards as well as unknown samples were measured with the help of digital vernier calipers. The concentrations (after $\log_2 n$ transformation) of the standards were regressed on diameter of the lysed zones around these standards. The slope of the curve and intercept were determined. The serum lysozyme and IgG concentrations in the unknown sera samples were estimated using the regression equation: $Y = bx + c$; where, Y, the concentration of serum lysozyme or diluted serum IgG in unknown sera sample; b, the slope of regression equation; c, the intercept of regression equation; and x, the diameter of the lysed zone around the unknown sera sample (Das *et al.* 2015).

Statistical analysis: The immunological data of each chicken line were analyzed by least square analysis of variance (Harvey 1990) incorporating sire as random and sex as fixed effects in the linear model: $Y_{ijk} = \mu + S_i + W_j + e_{ijk}$; where, Y_{ijk}, observation on k^{th} individual belonging to i^{th} sire and j^{th} sex; μ, population mean; S_i, random effect of i^{th} sire; W_j, fixed effect of j^{th} sex; and e_{ijk}, random error associated with mean zero and variance σ^2. The data after pooling over the chicken lines were also analyzed by least squares ANOVA taking sire within genotype as random, and genotype and sex as fixed effects in the statistical model. Genetic and phenotypic parameters were estimated for each chicken line using paternal half-sib correlation method (Becker 1975) taking sire as random and sex as fixed effects in the statistical model.

RESULTS AND DISCUSSION

The least squares means of the 3 immunocompetent traits i.e. haemagglutination (HA) titre, serum lysozyme and serum immunoglobulin-G (IgG) concentration are presented in Table 1. The least squares means of HA titre and serum IgG concentration were more in the control line than in the selected line, whereas the estimate of serum lysozyme concentration was more in the selected line.

The higher HA titre indicates presence of more total serum antibody *i.e.* immunoglobulins IgG, IgM, IgE, IgD and IgA produced till 5 dpi when the chicks were challenged with SRBC antigens; and the greater the titre value better the antibody response. The egg and developing embryo do not produce immunoglobulins until about 7 days before hatching (Jaiswal 2009). The hatchling is endowed with maternal IgG in the last few days before hatching (Kowalczyk 1985). It is therefore possible that egg has high lysozyme content to maintain vigilance until the embryo has developed the capacity to produce immunoglobulins. The lysozyme is also present in various body fluids (Jaiswal 2009). More the concentration of lysozyme in the body fluids, stronger the first line defenses to protect the birds being get infected by bacteria (Gram negative). More serum IgG concentration also indicates birds' better general immune response against any antigen. The present mean estimates of HA titre, serum lysozyme and serum IgG concentrations in the RIR chicken lines were in agreement to the earlier estimates in RIR chickens (Das *et al.* 2014a) and higher than the estimates in CARI-Debendra (Das *et al.* 2014b) and CARI-Sonali (Das *et al.* 2014c) crossbred chickens excepting serum lysozyme conc. in CARI-Sonali which was higher than in RIR control line. Present HA titre estimate in the selected line was comparable to the 5 dpi estimates reported in Dahlem Red chicken populations (Chatterjee *et al.* 2007) and White Leghorn chicken lines (Gupta *et al.* 2010). The present record for the HA titre in the control line was in approximation to the estimate reported in Aseel chicken (12.38±0.60) (Kumar and Kumar 2011). But the estimate for the serum lysozyme concentration in Aseel (Kumar and Kumar 2011) was not consistent with the present findings. The serum IgG concentration was consistent with the reports in a synthetic dam line of broiler chicken (Sivaraman *et al.* 2005), but

Table 1. The estimated least square means of various immunocompetent traits in the selected and control line of RIR chicken

Factor	Least squares means±standard errors					
	Haemagglutination (HA) titre		Serum lysozyme concentration (µg/ml)		Serum IgG concentration (µg/µl)	
	Selected line	Control line	Selected line	Control line	Selected line	Control line
Overall	8.854±0.359^b (79)	10.379±0.616^a (74)	6.503±0.591^a (74)	5.160±0.357^b (73)	6.663±0.455^b (74)	7.761±0.380^a (73)
Male	9.042±0.513 (41)	10.136±0.949 (35)	6.240±0.681 (38)	4.887±0.498 (35)	7.331±0.609 (38)	7.342±0.581 (35)
Female	8.665±0.530 (38)	10.622±0.905 (39)	6.766±0.687 (36)	5.433±0.483 (38)	5.995±0.618 (36)	8.179±0.561 (38)

Figures within parenthesis denote number of observations. Means with different superscripts in a row under a trait differed significantly ($P<0.05$).

lower than in the Aseel chicken (Singh *et al.* 2010). The differences in the estimates might be due to the different chicken breeds, strain or line studied.

The least squares analysis of variance revealed that sires of the selected line significantly (P<0.05) influenced the estimates of serum lysozyme and serum IgG concentrations; whereas the estimate of HA titre was not affected by sires in any line on contrary to the report in synthetic dam line of broiler chicken (Sivaraman *et al.* 2005).

The sex of the chicks did not affect (P<0.05) the estimates of any trait in any line in accordance to the previous reports (Das *et al.* 2014b, Das *et al.* 2014c, Gupta *et al.* 2010, Sivaraman *et al.* 2005). Although statistically non-significant, the means of HA titre and serum IgG concentration were higher in male birds than in the females, though the females had higher (P>0.05) means estimate of the serum lysozyme concentration in the selected line. But in the control line, all the estimates were higher in female birds than in the males. The sex could not follow any specific trend to influence the estimates of any trait in any line studied; although higher (P>0.05) estimates of the 3 immunocompetent traits (Das *et al.* 2014b) and first 2 ones (Das *et al.* 2014c) in the male birds than in the females were reported. These current results indicated that the genetic mechanisms responsible for mounting of antibody response to SRBC and regulation of the serum lysozyme and IgG levels might be sex-independent.

Analysis also revealed significant differences attributed by different genotype or line in the estimates of the three immunocompetent traits (Table 1). The birds of selected line contained more (P<0.05) serum lysozyme concentration than those of the control line, whereas the birds of the control line had higher estimates (P<0.05) of HA titre and serum IgG concentration. This line differences (P<0.05) in the estimates were in consistence with the reports in different genotypes (Das *et al.* 2014a).

The heritability and correlation estimates from sire component of variance are presented in Table 2. The 3 immunocompetent traits were heritable at a range of 0.105 to 0.398 in agreement to the earlier reports in a Dahlem Red chicken inbred population (Chatterjee *et al.* 2007) for the humoral immune response to SRBC (0.27±0.19); broiler chicken (Sivaraman *et al.* 2003) for the serum lysozyme (0.20±1.18); and Taiwan country chicken (Chao and Lee 2001) for the serum IgG concentration (0.307±0.217 to 0.418±0.175). The present low estimates of heritability indicated the presence of high environmental variances and the medium heritability estimates were indicative of greater role of additive genetic variance than the environmental component. Hence, selection may be useful for improvement of immunocompetence in these chicken populations. Falconer (1989) stated that heritability is a property of a trait of the population nourished by some environmental circumstances. Thus, any change in the components of variance will likely change the estimate of heritability and this may explain the attributed differences in the estimates by different workers. The heritability estimates may also be influenced by other factors not considered in the model used in this study and the estimated heritability in the present study were in the expected range.

The estimates of heritability of a trait can vary considerably from study to study depending upon breed, strain, line, population sampled, environmental and management conditions, and random as well as systematic errors in the estimation procedures (Mia *et al.* 2013). The data set from which these estimates were obtained was relatively small, and sampling errors were a consideration.

The three immunocompetent traits were correlated with a low to high range (0.134 to 0.552) of genetic correlation coefficients (r_G) and least to low range (0.007 to 0.170) of phenotypic correlations coefficients (r_P). The HA titre had positive and low estimates of r_G with the serum lysozyme concentration, and negative and high r_G with the serum IgG concentration in the selected line on contrary to the reports in a synthetic dam line of broiler chicken (Sivaraman *et al.* 2005), wherein HA titre had a positive and medium r_G with serum IgG, but non-precise r_G with serum lysozyme which was again reported with a non-precise r_G with serum IgG. But the present serum lysozyme concentration had a positive and moderate r_G with the serum IgG concentration in agreement to another report (Singh *et al.* 2010), though these estimates were associated with high standard errors (Singh *et al.* 2010, Sivaraman *et al.* 2005). Whereas, in the control line, the r_G between the HA titre and the serum lysozyme

Table 2. Estimated heritability (at diagonal), genetic (above diagonal) and phenotypic (below diagonal) correlations among various immunocompetent traits in the selected and control line of RIR chicken

Immunocompetent	Selected line			Control line		
traits	HA titre	Serum lyso conc.	Serum IgG conc.	HA titre	Serum lyso conc.	Serum IgG conc.
HA titre	0.105±0.458 (79)	0.184±0.454 (74)	–0.552±0.715 (74)	0.307±0.508 (75)	–0.321±1.261 (69)	NE
Serum lyso conc.	0.128 (52)	0.214±0.450 (70)	0.134±0.324 (74)	–0.170 (69)	0.312±0.430 (73)	NE
Serum IgG conc.	0.031(50)	0.033 (50)	0.398±0.589 (53)	–0.007 (69)	–0.038 (77)	0.223±0.459 (69)

Figures within parenthesis denote number of observations. HA titre, serum lyso conc. and serum IgG conc. denote haemagglutination titre, serum lysozyme concentration and serum immunoglobulin-G concentration, respectively; NE: Not estimated.

concentration was negative and non-precise due to high standard errors, which might be attributed to sampling variation owing to small sample size. The r_P among these immunocompetent traits were least to low in magnitude, and could not show any definite trend across the lines. Sivaraman *et al.* (2003) reported positive r_p between HA titre and serum lysozyme, and between serum lysozyme and IgG; and negative r_p between HA titre and serum IgG. Kean *et al.* (1994) also reported varied r_G and r_P among the three immunocompetent traits. The r_G between some immunocompetent traits could not be estimated in the control line, probably due to small number of observations per sire (>3) or sampling variation owing to small sample size in the present study. The lower and inconsistent estimates of r_P among the three immunocompetent traits had also been reported in various chicken breeds, strains or lines (Das *et al.* 2014b, Das *et al.* 2014c, Gupta *et al.* 2010, Sivaraman *et al.* 2005, Okada and Yamamoto 1987). Previously also, very low and non-significant correlations among these traits were reported in different chickens suggesting that these traits should be combined while selecting for improvement in general immune responsiveness (Van der Zijpp *et al.* 1983).

The present investigation concluded that the control line of RIR chicken might have better immunocompetence than the selected line. Sires might impact on the serum lysozyme and IgG levels of their progenies. The genetic mechanisms responsible for mounting of antibody response to SRBC and regulation of the serum lysozyme and the serum IgG levels might be sex-independent. The moderate estimated heritability indicated the presence of moderate environmental variances among these immunocompetent traits and possibility of selection of the birds to improve these traits that would not take propagation of long generations. The estimated correlations among these traits suggested that these traits should be combined while selecting birds for improvement in general immune responsiveness.

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