Genetic and non-genetic sources of variation in natural antibodies titre values among indigenous chicken

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Abstract

Innate immunity plays significant role in combating disease. Improvement of any trait including resistance to disease and infection requires identification of genetic and non-genetic sources of variation. This study aimed at deciphering the factors (both genetic and non-genetic) that confers variation in natural antibodies titres values in indigenous chicken of Kenya. The study was conducted at the Smallholder Indigenous Chicken Improvement Program Research Unit at Egerton University, Kenya. The meta data involving several factors (genotypes, ecotypes, cluster, sex, plate and breed) were analysed. The natural antibodies titre values were measured by indirect enzyme linked immunosorbent assay. Two sample t test for means and variance were compared for breed (RIR and IC) and sex (male and female) using IgG, IgA and IgM natural antibody isotypes titre values as dependant variables. One factor linear model was used to determine source of variation. A mixed model fitting chicken as random variable was used as the final model. For the two sample T test, there was significant difference on means for breed and sex (p=0.05) for all immunoglobulins isotypes. The T test showed significant difference in variance of breed (p=0.05) but not sex. However, the indigenous chicken and male chicken had higher variance estimates. Breed effect was significant for IgA (P=0.0323) but not IgG and IgM. Sex was significant for IgG (P=0.0279) but insignificant for IgA and IgM. Genotype and ecotype were insignificant while plate was significant respectively for both isotypes. Cluster was significant for IgA but not IgM and IgG. The variance estimate for chicken components were high and significant for IgM (p=0.003), IgG (p=0.0001) and IgA (p=0.0001). The residual variance estimates were small and insignificant for all the isotypes. The results implied the male had higher

NAbs titres values for all isotypes and could be used in selection of male line. The big variance estimate within the IC imply genetic improvement in NAbs against plethora of pathogens could be achieved through selection and crossbreeding.

Keywords: indigenous chicken, determinants, natural antibodies, variation

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Introduction

The chicken industry has been incurring substantial losses through mortality and morbidity due to infectious diseases worldwide (Lamont et al. 2010; van der Most et al. 2011). Conventional control of poultry diseases involves high-cost sanitary measures, biosecurity, vaccination programs, medical treatments and genetic resistance. Some of these control measures have antagonistic effect on genetic resistance to disease by allowing "unfit" animals into the breeding population, by preventing natural selection for genetic resistance to disease. Thus, breeding chickens for higher immunocompetence and disease resistance provides an alternative approach to securing chicken viability. Genetic resistance is defined as ability to resist any alteration of the state of the body by external causes (pathogens or stress) that interrupt or disturb proper performance (Lamont et al. 2010). It can be either resistance to infection, which will prevent the pathogen from becoming established in the animal, or resistance to disease, which prevents or reduces the development of pathological symptoms in animals infected with the pathogen. Genetic resistance is generally assumed to be considerable varied among breeds with respect to their ability to withstand and survive pathogenic infections (Rahman et al. 1997). In large-scale commercial poultry production, it

is common to vaccinate against pathogens that may harm the birds. In contrary, smallholder farmers regularly face financial constraints that make vaccination difficult to implement (Okeno et al. 2011). It is important that chicken kept under free-range systems, a system predominant in developing countries, have sufficient heritable robustness to withstand the high levels of disease challenge to which they are exposed to. With this regard, genetic resistance was ranked a trait of economic importance for indigenous chicken (IC) in Kenya (Okeno et al. 2011) that should be included in the breeding goal. Reports on the performance of IC in developing countries suggest that they are resistant to diseases (Rahman et al. 1997) and produce more acquired antibodies to Newcastle virus (Lwelamira et al. 2004) than exotic birds. This trait can be exploited for genetic improvement.

Improvement of any trait including resistance to disease and infection requires that genetic and non-genetic sources of variation are identified. This is because non-genetic sources influence the expression of genetic potential for traits and provide a better understanding of biological or environmental mechanisms on performance (Mrode, 2005). Similarly, accounting for these factors help to unmask true differences between groups as well as reduce bias in performance evaluation. Several studies on production traits done on IC have been reviewed and genetic and environmental factors found to influence the traits. The evidence outlined in these reviews majored on productive traits and with scanty information on disease resistance and immune response (Khobondo et al. 2014). The two branches of immunity (innate and specific) are important in combating diseases (Beutler, 2004). Innate immunity plays an important role in survival of organisms, although additionally acquired immunity is often required in vertebrates (Beutler, 2004). Natural antibodies (NAbs) are part of innate immunity, found in healthy unimmunized individuals and are an important part of the first line of defense in animals. They provide early resistance against plethora of infections (Ochsenbein and Zinkernagel, 2000) because of their polyspecificity and polyreactivity. Low

levels of innate immunity (NAbs) may be related with enhanced disease susceptibility and high levels with disease resistance and high survival rate (Sun et al. 2011) in commercial chicken breeds. In contrary, under intensive production system (modern housing systems) the (opposing) relationship between NAb at early age and survival has been reported for commercial (elite) layer breeds (Star et al. 2007; Sun et al. 2011). Recently, high NAb levels have been associated with low survival in IC breeds in Ethiopia (Wondmeneh et al. 2015). That finding suggested that NAbs have a positive effect on survival in adapted intensively reared chicken (exotic breeds) but that effect was reversed in IC that are not adapted to confinement. These findings on NAbs, however, fall short of the factors determining their variation in chicken. Studies using IC in Kenya using quantitative genetics methods highlighted differences in several production traits (Khobondo et al. 2015). Molecular studies of diversity on the same population have grouped the chicken into three phylogenetically distinct clusters (Ngeno et al. 2014). It is not however known whether differences in Nabs titres exist between these clusters (Ngeno et al. 2014), genotype (Magothe et al. 2012) and ecotypes (Okeno et al. 2013) of IC as outlined elsewhere (Khobondo et al. 2014). Therefore, current study aimed at determining the sources of variation in Nabs titre values traits of IC.

Materials and Methods

Study Site

The study was conducted at the Smallholder Indigenous Chicken Improvement Program Research Unit (INCIP-RU) at Egerton University, Njoro, Kenya. The University lies on 35°45'- 35°46' E and 0°16' - 1°10' S, at an altitude of 1800 meters above sea level with an average annual rainfall of about 1000mm and mean temperature range between 17-22°C (Ayuya et al. 2011). The area lies in agro-ecological zone one of Kenya (Sombroek et al. 1982).

Experimental Population

The base population of chicken used in this study was established through collection of chicken and eggs from unselected, random mating population of IC from the rural farmers of Kakamega, Bondo, West Pokot, Narok, TaitaTaveta, Lamu and Bomet counties in Kenya thus, called ecotypes. The counties were chosen because there had no introduction of exotic chicken before hence minimum genetic dilution of the IC were expected. From these chickens and eggs, a population of IC was established on station at INCIP-RU at Egerton University.

The ecotypes were genotyped and the population originating from various counties in Kenya clustered into three distinct phylogenetic groups as described by Ngeno et al. (2014). Cluster one constituted chicken from the Western, North and South Rift Kenya (Kakamega, Bondo, West Pokot, Narok, and Bomet), cluster two constituted chicken from the Eastern region and cluster three constituted chicken from the coastal region in Kenya (Lamu, Taita Taveta) (Ngeno et al. 2014). Sex was determined by phenotypic appearance. Genotypes (naked-neck, normal feathered, frizzle genes, Kuchi) were described on phenotypic observation.

Health and Disease Management

The chicken received routine vaccinations against Marek's disease (day 1), New Castle disease (NCD; week 3, 8, 18), infectious bursal disease (week 2, 7) and fowl typhoid (week 8).

Management

At hatch, each chick was wing-tagged and identified by the ecotype, cluster, genotype, sex and breed. Brooding was done from hatching to 8 weeks in deep litter brooders that were heated using infrared bulbs. The population density was 12chicks/m^2. The chicks were fed rations with nutrient composition recommended for IC in confinement. The chicks were

provided with a chick feed (20% CP and 2,950 kcal/kg) for 8 weeks after hatching. Twenty-two hours to 23 hours light was provided during the first 3 days and 10 hours light afterwards for 8 weeks. Thereafter, natural lighting was used as the day length was more or less the same in Kenya during the study period. Infrared lamp of 250W was used to provide heat. The temperature during the first 3 days was 28°C to 30°C and was reduced to 23°C in the 4th week. Afterwards, 23°C to 25°C was provided throughout the study period. The temperature was monitored using thermometer and ventilation was adjusted by opening the curtains.

Thereafter the chicks were fed on grower ration (18%CP and 2,850 kcal/kg) from week 9 to 17. From 18 weeks onwards the chickens were provided with ad libitum layer mash (16% CP and 2,750 kcal/kg) feed. Clean water was provided ad libitum. Health management practices such as vaccination, deworming and disinfection were carried out procedurally. The chickens were kept in open house filled with wood shavings on concrete floor and deep litter. Standard density of 8 and 6 chickens/m^2 were used during the rearing and laying period respectively.

Natural Antibodies Isotype Assays

Blood samples (2 ml in EDTA) from 215 chickens of between 45 to 49 weeks were drawn from the wing vein of each chicken and plasma separated by centrifugation at 2000 rpm for 10 minutes. Isotype specific IgA, IgM and IgG antibody titers to keyhole limpet hemocyanin (KLH) in plasma from the chicken were determined by indirect enzyme-linked immunosorbent assay (ELISA). Keyhole limpet hemocyanin (KLH) is a high-molecular-weight protein antigen collected from the hemolymph of the sea mollusk (Megathura crenulata). It is a copper-containing high-molecular-weight protein, which is commonly used as a soluble model protein known to induce a T-helper 2 cell (Th2)-like response (Bliss et al. 1996). This antigen has been used in several studies of chicken immunity (Minozzi et al. 2008) and QTL studies based on microsatellite markers (Siwek et al. 2003). Keyhole limpet

hemocyanin is an antigen that birds have not encountered during their lifetime; therefore, it represents a novel antigen, suitable to measure primary immune responses. Briefly, 96 well plates were coated with 2µg/ml KLH (MP Biomedicals Inc., Aurora, OH) and incubated overnight at 4°C. The plates were washed five times with 100 µl using a washing/dilution buffer (phosphate buffered saline (PBS) containing 0.05% Tween) and incubated for 1.5 hours at 25°C with chicken plasma (in duplicate) diluted 1:10 in dilution buffer. The plates were washed five times to remove unbound plasma. To detect IgA, IgM and IgG antibodies binding to KLH, a secondary 1:20,000 diluted affinity purified goat anti-chicken IgM (Fc specific), conjugated with horseradish peroxidase (GACh/IgM (Fc) /PO) antibody, or 1:20,000 diluted whole rabbit anti-chicken IgG (heavy and light chains) conjugated with horseradish peroxidase (RACh/IgG (H+L)/PO) antibody or 1:20,000 diluted affinity purified goat anti chicken IgA (Fc specific) conjugated with horseradish peroxidase (GACh/IgA (Fc) /PO) (Nordic Immunological Laboratories, Eindhoven, The Netherlands) was added and incubated for 1.5 hours at 25°C. The plates were washed five times again and 100µl substrate-buffer (containing aqua dest, 10% tetramethylbenzidine-buffer and 1.33% tetramethylbenzidine) per well was added and incubated for 10 minutes at room temperature in darkness. The reaction was stopped with 1.25M H_2SO_4. Absorbance levels were measured with a spectrophotometer (mrc Scientific Instrument-UT- 6100, Israel) at 620 nm.

Statistical Analysis

Means and Variance Analysis for Sex and Breed

Two sample t test for means and variance were compared for breed (RIR and IC) and sex (Male and Female) using IgG, IgA and IgM Nabs titre values as dependant variables.

Factors influencing Antibody titres values

A step wise analysis to determine factors influencing variance was done using PROC GLM procedure of SAS 9.1 (SAS, 2002). Finally, a mixed model analysis of variance on antibody titre values traits was carried out to determine the factors that influence variation using PROC MIXED procedure of SAS 9.1 (SAS, 2002). The independent variables fitted included breed, sex, ecotype, cluster, genotype and plate. The identity of chicken was fitted as random. The mixed model used for the analyses is presented below:

$$Y_{ijklm} = \mu + S_i + G_j + C_k + B_l + Pm + ECn + A_k + e_{ijlkm}$$

where: Y_{ijklm} is the performance trait of the m^{th} bird; μ the overall mean; S_i the effect of i^{th} sex (i = male, female); G_j the effect of j^{th} genotype (j = normal feather, naked-neck, frizzled, kuchi); C_k the effect of k^{th} cluster group (k = CL1, CL2, CL3, CL4); B_l the effect of l^{th} breed (l = IC, EX); Pm the effect of m^{th} plate (m=1,2,3); ECn the effect of n^{th} ecotype and A_{ki} the random effect of the chicken and e_{ijklm} the random error term.

Results

Comparison of Mean and Variance Components between Sex and Breed

The difference in mean and variance estimates between sex (M/F) and breed (Exotic/ IC) was determined for all isotypes. In all the isotypes, males had significantly higher means than females (P≤0.05). For IgG isotype, there was significant difference in variance estimate (P=0.0404) for breed effect with the IC having relatively higher variance (Table 1). However, the variance estimates for sex and breed was insignificant for IgM and IgA. For the same isotypes, however, IC had higher variance estimate than exotic breed.

Table 1. The two sample T test for variance showing significant difference in variance of breed but not sex. The IC and male chickens have higher variances

Isotype	Group/Factor	Breed	Number	Mean	Variance	Pvalue (variance)
IgG	Breed	Exotic	21	2.3216	0.1501	0.0404
		Indigenous	194	2.1905	0.3318	
	Sex	Female	128	2.1496	0.3308	0.1118
		Male	71	2.3405	0.2399	

Determinant of Antibody titre values

The one way ANOVA for IgA, IgG and IgM revealed significant and insignificant effects. Breed effect was significant for IgA (P=0.0323) but not IgG and IgM. Sex was significant for IgG (P=0.0279) but insignificant for IgA and IgM. Genotype and ecotype were insignificant while plate was significant respectively for both isotypes. Cluster was significant for IgA but not IgM and IgG.

The mixed model analysis showed significant difference in chicken for all isotypes. The variance estimate for chicken (random variable) components were high and significant for IgM (p=0.003), IgG (P=0.0001) and IgA (P=0.0001). There was a big variance estimate among the chicken as compared to residual variance. The residual variance was insignificant (Table 2).

Table 2: The variance estimates for chicken (genetic) and residual and their significance levels for IgA, IgG and IgM Nabs titre values. The number of stars shows level of significance

	Variance Estimates		
	IgM	IgG	IgA
Source of variation			
Chicken	0.01795***	0.2544****	0.1164****
Residual	0.00414	0.1068	0.0098

Discussion

The study aimed at demystifying the variation of NAbs titre levels for three isotypes in IC in Kenya. Natural antibodies were found to be detectable in the IC population (Khobondo et al. 2016), similar to reports of non-immunized cattle (Van Knegsel et al. 2007), humans (Ehrenstein and Notley, 2010) and poultry (Sun et al. 2011). In mammals, the NAbs are mostly produced by CD5+ B cells in the peritoneal cavity and intestines but also CD5- B cells (Casali and Notkins, 1989) were described to produce NAb. NAbs may arise independently of known antigenic stimulation. They are mostly poly-reactive, and poly-specific (Baccala et al. 1989) with low binding affinity hence confer general immune competence (Casali and Notkins, 1989). Different isotypes of Nabs (IgM, IgA and IgG) have been reported to be present in many animals, which is in agreement with the findings of this study. The study also confirmed that the IgM isotype is the most abundant whereas IgG and IgA Nab are relatively higher titres as explained elsewhere (Khobondo et al. 2016).

The breed difference for IgG (table 1), cluster difference for IgA and within chicken difference for all isotypes (table 2) observed in this study is in consistent with other studies which showed that NAbs are genetically controlled (Sun et al. 2011). The genetic clusters used in this study were initially determined by Ngeno et al. (2014) using MHC linked markers. Two main population clusters using MHC linked markers indicated by ΔK and PCoA were Lamu (one cluster) and populations from Kakamega, West Pokot, Turkana, Bomet, Narok and Siaya as a second cluster. An extra group (third cluster) was from Taita-Taveta. This distinction was only existent with regard to IgA Nab titres isotype. The two sample T test for variance analysis of breed (Exotic/IC) explained significant breed difference for IgG isotype. In this analysis the IC had higher variance estimates than RIR implying that more genetic response can be achieved by selection within IC population. The large variance components conferred by IC in the mixed model showed individual variation

within the chicken. This high variance estimated among chickens suggested NAbs can be used for breeding for robustness that is a broad capacity to remain healthy across a wide range of challenges. A study using acquired immunity against Newcastle disease virus (NDV) involving local chickens from Tanzania (LL), Rhode Island Red derivative hybrids (EE), and their reciprocal crosses (LE and EL) showed immune response differences. The local ecotype had the highest mean antibody titres following primary and booster vaccination while the exotic breed had the lowest titres (Lwelamira and Katule, 2004). In another study, there was significant difference in NAbs titres values on layers. The chickens in that study were from three different lines, which were either divergently bred during 29 generations for high (H line) or low (L line) primary (agglutinating) antibody responses at day 5 after primary intramuscular immunization with Sheep Red Blood cell (SRBC) at 37 days of age, or a random bred control (C line) (Parmentier et al. 2004).

Sexual dimorphism has been reported to influence other traits, for example weight at 12 weeks (Muasya et al. 2015) and growth. Breeding program in poultry is carried out in the nucleus stock (pedigree stock). In three to five generations, the genetic improvement is then forwarded through multipliers to commercial farmers. In this study, male chicken had higher Nabs values as compared to females for all isotypes. The results can be exploited in IC breeding where a terminal product is crossed between male and female lines. The male line can be used for NAbs traits for increased heterosis. For example, in breeding for meat producing chicken, male lines could be selected for NAbs titres, growth and conformation and crossed with female lines selected for reproductive characteristics.

The genotype of IC were reviewed and reported to affect productive traits (Khobondo et al. 2014). Variations on body weights among the genotypes, and estimation of heritabilities and genetic correlations in IC in Kenya were reported (Magothe et al. 2012). Furthermore a study on the same population showed that body weights and growth patterns are influenced by

genotypes (the naked-neck, frizzle and crested-head) (Magothe et al. 2012). The previous studies demonstrated that the crested-head genotype had a slower growth rate and were lighter compared to the normal-feather genotype when subjected to the same level of management. Furthermore, genotypes (both exotic and indigenous) possessing the naked-neck and frizzle genes, either singly or in combination were associated with increased growth rates, superior body weights, better feed conversion, higher egg production and disease tolerance in tropical environments (Islam and Nishibori, 2009). The Kuchi genotype, found in Tanzania (Msoffe et al. 2002) and similar to the Aseel genotype of Bangladesh (Bhuiyan et al. 2006) had higher growth rate and would be ideal for meat production in warm and humid areas. This distinction has, however, no influence on NAbs titre values.

The effect of the micro titre plate was found to be significant in this analysis. This observation could be attributed to intrinsic value and properties of each ELISA plate. The effect of plate was observed in another study (van der Klein et al. 2015) where the plate effect was corrected for other (confounding) effects on the samples, such as sex, storage, and analyses effects. It not yet established whether these effects could apply in this study and thus it worth to investigate further.

Conclusions

The study confirmed significant variation of NAbs on the IC in Kenya. Breed and cluster (both genetic) were significantly different in IgA NAbs titre values binding KLH. The male IC had higher NAbs titres values for all isotypes and could be used in selection of male lines for breeding for high natural antibody titres. There was a big variance within the IC, it is proposed that substantial genetic improvement in NAbs against plethora of antigens could be achieved through both selection and crossbreeding. These strategies could alter allele frequencies of genes and markers linked to natural antibody titres.

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Disclosure statement

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

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