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Polymorphism of TLR7 Gene in the Nigerian Indigenous Chickens and ISA Brown Commercial Layer Chicken

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Abstract
This study was designed to examine the genetic polymorphism of TLR7 gene in the Nigerian indigenous chicken populations and ISA Brown commercial layer chicken. The study population comprises eight (8) chicken groups (naked neck, normal and frizzled-feathered Nigerian indigenous chickens in rain forest and Guinea Savannah regions respectively, Fulani ecotype chicken and ISA Brown commercial layer chicken). Blood samples were collected from each of the eight chicken groups. Genomic DNA was isolated from each blood sample using the Zymo Quick-gDNA™ Miniprep kit (D3024, Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's instructions. The DNA sequencing of chTLR7 gene was done using the Sanger Sequencing Chemistry.26 novel single nucleotide polymorphisms (SNPs), two deletions and two insertions in the intronic region of TLR7 gene in the Nigerian indigenous chicken population and ISA Brown commercial layer chicken were found. It is, therefore, recommended that further research should also be carried out on SNP genotyping to determine the association of SNPs of TLR7 gene with resistance or susceptibility to infections in the Nigerian indigenous chickens.

Keywords*: Chicken, diversity, intronic region, indels, mutations, Toll-like-rceptor.*

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I. Introduction

In Nigeria, as well as other countries in the world, native chickens constitute indispensable animal genetic resource (AnGR) from which several lines of chicken perhaps have been developed. Nigerian as a tropical nation has different vegetation types such as rainforest zones of South-east, South-west and South-south; Guinea Savannah zones of North-central, North-east and North-western Nigeria. The Nigerian indigenous chickens found in each of the ecological zones perhaps constitute different genetic populations with restricted inter-population gene flow, which could be due to geographical isolation (natural factor) or artificial boundaries (Ukwu *et al*., 2017). These nativechickenstherefore may have evolved adaptabilities in their local environment. Thus, it is paramount to assess the genetic diversity of the native chickens in Nigeria over time, since genetic diversity is important for the perpetuation of a species.

Genetic analysis of Nigerian local chicken populations using molecular genetics techniques could uncover any DNA based polymorphism existing among the indigenous chicken populations across different ecological regions in Nigeria. Polymorphisms at the level of the DNA can be useful markers for molecular characterization of indigenous chickens. If such polymorphisms at the level of the gene or DNA are uncovered, they can be exploited in marker-assisted selection (MAS) and gene-assisted selection (GAS) for the purpose of genetic improvement of Nigerian indigenous chickens. Genetic characterization is also useful to help conserve the valuable genetic variants inherent in the indigenous chicken genetic resource.

Although some researchers have attempted genetic study of Nigerian indigenous chickens using molecular genetics approach (Ohwojakpor *et al*., 2012; Illori *et al*., 2016; Adebambo *et al*., 2010; Ajibike *et al*., 2017), there is dearth of information on polymorphism of chicken TLR7 gene in the Nigerian indigenous chickens and ISA Brown commercial layer chicken.

Toll-like receptor 7 (TLR7) gene is a member of multi-gene family, which is implicated in the recognition of viral pathogen-associated molecular patterns (PAMPs) (Jensen and Thomsen, 2012). Since toll-like receptors 7 (TLR7) gene is implicated in intracellular recognition of nucleic acids, especially viral ribonucleic acid (RNA), and has been sequenced, therefore, there is need to exploit this gene in the Nigerian local chicken populations and ISA Brown commercial layer chicken in order to uncover hidden functional

variants that may exist at TLR7 locus. Such functional variants at the level of the DNA, if uncovered, could be exploited in further studies of disease resistance and also used as molecular markers in genetic selection for the purpose of improvement of quantitative traits in the Nigerian local chickens.

Research reports have shown that most studies involving conventional sequencing are usually restricted to exons and exon-intron boundaries because it is believed that the introns are completely eliminated during RNA processing through the process of splicing. But recent research information emanating from entire genome sequencing and analysis of mRNA have revealed that mutations associated with some diseases can occur deep inside the intronic regions of a gene, which are not detected by conventional sequencing delimited to exons and exon-intron boundaries (Wang *et al*., 2015; Vaz-Drago *et al*., 2017; Mendes de Almeida *et al*., 2017). For example, deep intronic mutation (c. 639+919G>A) in the galactosidase alpha (GLA) gene has been reported to be implicated in pseudoexon activation, Fabry disease, by disrupting the binding of hnRNP A1 and hnRNP A2 to an exon splicing silencer (Palhais *et al*., 2016). Another report has also shown that full genomic DNA sequences of vinculin (VCL), protein kinase AMP-activated non-catalytic subunit gamma2 (PRKAG2) and titin (TTN) genes revealed potential pathogenic deep intronic variants, which are predicted to act through disruption of either splicing or transcription factor binding sites (Mendes de Almeida *et al*., 2017).Therefore, we hypothesize that some base changes in the introns and few critical bases at the splice recognition sites of TLR7 gene could result in failure to splice out the introns or aberrant splicing and consequently result to a severely damaged protein product when the mis-spliced mRNA is translated (Clark, 2005).

This study, therefore, was designed to determine polymorphisms within the intronic region of TLR7 locus in the Nigerian indigenous chickens and ISA Brown commercial layer chickens.

II. Materials And Methods

Blood sample collection

*About 1 ml of*whole blood sample from each bird was aseptically collected from the brachial vein, separately into vaccutainer tubes (SARSTEDT Monovette®) containing Ethylene-diamine-tetra-acetic acid (EDTA) as anticoagulant, using sterile needles. Blood samples were collected from indigenous chickens from rainforest region, Guinea Savannah region, Fulani ecotype chicken, and ISA Brown commercial layers chickens.

DNA extraction and integrity

Genomic DNA was isolated from each blood sample using the Zymo Quick-gDNA™ Miniprep kit (D3024, Zymo Research Corporation, Irvine, CA, USA) following the manufacturer's instructions. The kit is a unique extraction technology for easy quick isolation of ultra-pure DNA from whole blood (and other DNA sources) in less than 15 minutes, using clean spin-column technology. The concentration and purity of each DNA sample was determined using Nanodrop Spectrophotometer.

Polymerase Chain Reaction (PCR) and sequencing of TLR7 gene

After DNA extraction and quantification, three (3) DNA samples from each genetic group (or breed) were taken for TLR7 genes discovery and polymerase chain reaction (PCR). The primers used for PCR amplification of target gene (chTLR7) were designed using the Primer3 and BLAST option at the NCBI database (www.ncbi.nlm.nih.gov). The information on primers used is presented in Table 1. Polymerase chain reactions (PCR) were performed in a 50 µl reaction volume containing 10 µl of 5X FIREPol® Master Mix (Solis BioDyne, Tartu, Estonia), 2.5 µl each of forward and reverse primers, 31 µl of nuclease-free water and 4 µl sample DNA template. FIREPol® Master Mix reagent composition includes FIREPol® DNA polymerase, 0.4 M Tris-HCl, 0.1 M $(NH_4)_2SO_4$, 0.1 % w/v Tween-20, 12.5 mM $MgCl_2$, 1 mM dNTPs (200 µM each of dATP, dCTP, dGTP, dTTP), blue dye, yellow dye and compound that increases sample density for direct loading. PCR conditions consist of 1 cycle of 95°C for 4 minutes initial denaturation, 35 cycles each of 95°C for 30 seconds denaturation, 62°C for 30 seconds annealing, 72°C for 1 minute elongation, followed by 72°C for 10 minutes final elongation.The DNA sequencing of chTLR7 gene was done with the same PCR primers using the Sanger Sequencing Chemistry, and Q20 read length up to 800 bases with ABI file were reported.The identification of single nucleotide polymorphisms (SNPs) of the chTLR7 gene in the Nigerian indigenous chickens and ISA Brown commercial layer chickens was done using GenAlEx 6.502.

Table 1: Primers and sequences for amplification of TLR7

Primer	Primer sequence	GC Content %
TLR7-F	AGGCTTGCTGTTGTGGCATGGA	54.55
TLR7-R	ACTGGAAGCCCTTCCTCCACTGT	56.52

III. Results And Discussion

Analysis of 521-bp fragment of intronic region of the TLR7 gene sequences from the Nigerian indigenous chickens, ISA Brown commercial layer chicken and the reference sequence (Red jungle fowl; Accession: NC_006088.5) revealed 496 conserved sites, 25 variable sites, 11 singleton sites and 14 Parsimony informative sites. The result also showed that 95.2% of 521-bp fragment of TLR7 gene were conserved.

Single Nucleotide Polymorphisms (SNPs) and Indels of TLR7 gene in the Nigerian indigenous chickens and ISA Brown commercial layer chicken

Single nucleotide polymorphisms (SNPs) and Indels of TLR7 gene in the Nigerian indigenous chickens are presented in Table 2. The result of this study revealed a total of 30 single base variations comprising 26 novel SNPs, and four Indels in the intronic region of TLR7 gene sequences from the Nigerian indigenous chickens and ISA Brown commercial layer chicken.

The chTLR7 novel SNPs shared among the Nigerian indigenous chickens and the ISA Brown commercial layer chicken were: A>C (position: A1711C, A1723C), A>G (positions: A1691G, A1694G, A1821G, A2139G), C>G (position: C1731G), G>A (position: G1788A), T>A (position: T1703A), T>C (position: T1798C, T2197C).

The chTLR7 novel SNPs unique to the Nigerian indigenous chickens were: A>G (position: A1701G, A1702G), A>T (position: A1714T), C>A (position: C1720A), C>G (position: C1698G, C1763G), C>T (position: C1720T, C1951T, C2029T), T>G (position: T1706G, T2173G, T2175G), T>C (position: T1717C, T1727C), G>C (position: G2203C).

The Indels, which were unique to the Nigerian indigenous chickens, were: insertion of 'T' at chTLR7 position 2174, insertion of 'C' at chTLR7 position 2196, deletion of 'T' at chTLR7 position 1730 and deletion of 'C' at chTLR7 position 1731.

In this study, we report the first investigation on the chTLR7 gene variations in the Nigerian indigenous chickens and ISA Brown commercial layer chicken. Although the chTLR7 gene has been reported to be highly conserved when compared to mammalian TLR7 gene (Yilmaz *et al*., 2015), our analysis of the chTLR7 gene in the Nigerian indigenous chickens and ISA Brown commercial layer chicken revealed several mutations at different positions in the intronic region. It has been reported that chTLR genes are polymorphic among different chicken breeds, suggesting a varied resistance among several breeds of chicken (Ruan *et al*., 2015). It has earlier been reported that although avian TLRs display low to moderate single nucleotide polymorphism levels, TLRs seem to be more polymorphic in free-ranging populations of birds than in livestock (Alcaide and Edwards, 2011). Polymorphisms existing in TLRs may have strong influence on host responses to variety of viruses and hence may be associated with susceptibility or resistance to diseases (Misch and Hawn, 2008). Perhaps, polymorphisms found at the chTLR7 gene can be exploited in association studies to identify chicken genotypes or breeds with genetic potential for resistance to diseases such as avian influenza, infectious bursal disease etc.

Analysis of the TLR7 gene sequences from the Nigerian indigenous chickens and ISA Brown commercial layer chicken revealed 26 SNPs and four (4) INDELs located in intronic region. Over the years, sequencing information has been restricted to exons and exon-intron boundaries. However, recent reports emanating from mRNA analysis and entire genomic sequencing have shown that mutations associated with some diseases can occur deep inside the intronic regions of a gene, which are not detected by conventional sequencing delimited to exons and exon-intron boundaries (Vaz-Drago *et al*., 2017; Mendes de Almeida *et al*., 2017). For example, the existence of SNPs in the intronic region of Piwi gene has been reported in quail and a few breeds of chicken in China (Wang *et al*., 2015). Again, some base changes in an intron and few critical bases at the splice recognition sites could result in failure to splice out the intron or aberrant splicing. This could consequently result to a severely damaged protein product when the mis-spliced mRNA is translated (Clark, 2005).

Again, recent reports have also shown that intronic mutations can affect RNA processing through exon skipping, cryptic splicing, intron inclusion, leaky splicing and introduction of pseudo-exons into the processed mRNA (Holla *et al*., 2009; Nascimbeni *et al*., 2010; Caminsky *et al*., 2014). Intronic mutations and indels in the regulatory regions can affect gene expression by introducing or eliminating enhancer activity, introducing novel splice sites or novel promoters. It has been reported that mutations that occur deep inside introns can disrupt transcription regulatory motifs and non-coding RNA genes (Vaz-Drago *et al*., 2017). It has further been reported that deleterious DNA variants located more than 100 bp away from exon-intron junctions often lead to pseudo-exon inclusion due to activation of non-canonical splice sites or changes in splicing regulatory elements (Vaz-Drago *et al*., 2017). Although there are no reports on SNPs and INDELs in the intronic region of chTLR7 gene, our study is the first to report intronic mutations and INDELs in the TLR7 gene in the Nigerian indigenous chickens and ISA Brown commercial layer chicken.

Table 1: SNPs and Indels of chTLR7 in the Nigerian indigenous chickens and ISA Brown layer chickens

Number	Position on TLR7	SNP	Chicken
1	1691	A>G	Gn IS Rn
2	1694	A>G	Gn IS
3	1698	C>G	Gn
4	1701	A>G	Rn
5	1702	A>G	Gn
6	1703	T>A	Gn IS
7	1706	T>G	Gn
8	1711	A>C	Gn IS Rn
9	1714	A>T	Gn
10	1717	T>C	Gn
11	1720	C>A C>T	Gn RN
12	1723	A>C	Gn IS Rn
13	1727	T>C	Gn
14	1730	deletion of T	FE Gn

Table 1: Continued

Number	Nucleotide Position	SNP	Chickens
15	1731	Deletion of C C>G	FE Gn Gn IS
16	1763	C>G	GF RN
17	1788	G>A	FE GF Gn RN Rn IS
18	1798	T>C	FE Gn IS RN
19	1821	A>G	FE GF GN Rn Gn IS RN

20	1951	C>T	GF RN
21	2029	C>T	GF
22	2139	A>G	FE GN Rn Gn IS RN

Table 1: Continued

Number	Position	SNP	Chickens
23	2173	T>G	GF
24	2174	Insertion of T	GN RF
25	2175	T>G	GF GN RF
26	2196	Insertion of C	GF RN GN
27	2197	T>C	FE Gn IS Rn
28	2203	G>C	GF

Gn= guinea savanna normal chicken, GF= guinea savannah frizzle-feathered chicken, GN= guinea savannah naked neck chicken, Rn= rain forest normal chicken, RF= rain forest frizzle-feathered chicken, RN= rain forest naked neck chicken, FE= Fulani ecotype chicken, IS= ISA Brown commercial layer chicken

IV. Conclusion

The results of this study revealed 26 novel single nucleotide polymorphisms, two deletions and two insertions in the intronic region of TLR7 gene in the Nigerian indigenous chicken population and ISA Brown commercial layer chicken. It is, therefore, recommended that further research should be carried out on SNP genotyping to determine the association of the mutations of TLR7 gene with resistance or susceptibility to infections and some quantitative traits in the Nigerian indigenous chickens. The SNPs of TLR7 gene could be used as molecular markers in marker-assisted selection to produce chickens lines with good antiviral response.

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