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Molecular basis of susceptibility to Newcastle disease virus challenge in exotic and local Tanzanian chicken

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MOLECULAR BASIS OF SUSCEPTIBILITY TO NEWCASTLE DISEASE VIRUS CHALLENGE IN EXOTIC AND LOCAL TANZANIAN CHICKEN

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ABSTRACT

This research aimed to investigate genetic mechanisms of susceptibility to Newcastle disease virus (NDV) in exotic and local Tanzanian chicken. In the first experiment kuroiler, broiler and local Tanzanian chickens were vaccinated with live La Sota Newcastle disease (ND) vaccine, and body weight gain and antibody responses were used as phenotypes to evaluate chicken susceptibility to NDV. Results showed higher ($P < 0.05$) antibody titres in kuroilers (3.81 ± 0.06) as compared to local chickens (3.73 ± 0.07) and broilers (3.53 ± 0.06) at day 10 post-vaccination. However, antibody titres were not different ($P > 0.05$) between kuroilers and local Tanzania chickens at day 21 post-vaccination. Although results showed differences between vaccinated and control groups, the results could not give clear cut differences on variations in susceptibility, probably because a less virulent strain of NDV was used and the housing environment might have created some confounding variables. Therefore, in the second experiment virulent NDV and chicken embryo model were used to investigate chickens variation in susceptibility to NDV where time of death post-challenge was used as a phenotype. A total of 355 (87 Sasso, 129 kuroiler and 139 local) 16-day-old chicken embryos were challenged with virulent NDV, and death time was recorded post-challenge. Candidate gene and selective genotyping approaches were deployed, and therefore, chicken embryos from high (15%) and less (15%) susceptible cohorts were genotyped for selected genes (myxovirus resistance gene (Mx) and LEI0258). As expected, chicken embryos survival time was highly variable within a breed. Furthermore, it was demonstrated that chicken Mx gene G2032A genotypes (AA, AG, and GG) were associated ($P < 0.05$) with susceptibility. Interestingly, for the first time, findings demonstrated an association between chicken Mx gene promoter polymorphisms and chicken embryos susceptibility to virulent NDV. Specifically, SNP4 G>A mutation located within IFN-stimulating response element was associated (LR: 6.97, $P = 0.03$) with susceptibility. Also, haplotype ACGC was associated (OR: 9.8, 95% CI: 1.06 – 79.43, $P = 0.042$) with the same trait, and had a protective effect. The present findings are very useful in breeding programs designed to develop chicken genotypes, which are less susceptible to NDV.

DECLARATION

I, Fulgence Ntangere Mpenda do hereby declare to the Senate of the Nelson Mandela African Institution of Science and Technology that this thesis is my own original work and that it has neither been submitted nor being concurrently submitted for degree award in any other institution.

Fulgence Ntangere Mpenda

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The above declaration is confirmed

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Name and signature of supervisor 2 **Date**

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CERTIFICATION

This is to certify that this thesis titled "Molecular basis of susceptibility to Newcastle disease virus challenge in exotic and local Tanzanian chicken" is written by Fulgence Ntangere Mpenda under supervision of Prof. Joram Buza and Prof. Sylvester Leonard Lyantagaye at the NM-AIST. I approve the thesis for submission to the NM-AIST senate for the award of the PhD degree in Life Sciences.

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Name and signature of supervisor 2 **Date**

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DEDICATION

This work is dedicated to my parents; my father Mpenda Mlebeke and my mother Katalina Mbanga.

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ABBREVIATIONS AND SYMBOLS

ACGG	Africa Chicken Genetic Gains
ANOVA	Analyis of Variance
BP	Base Pair
BWG	Body Weight Gain
cDNA	Complementary Deoxyribose Nucleic Acid
CEs	Chicken Embryos
cRBCs	Chicken Red Blood Cells
DNA	Deoxyribose Nucleic Acid
DT	Death Time
ELISA	Enzyme Linked Immunosorbent Assay
F	Fusion
GH	Growth Hormone
GTP	Guanosine Triphosphate
GVs	Genetic Variants
HA	Hemaglutination Test
HI	Hemaglutination Inhibitioin Test
HN	Hemagglutinin Nuraminidase
IFN	Interferon
ISRE	Interferon Stimulating Response Element
LD	Linkage Disequilibrium
LDLR	Low Density Lipoprotein Receptor

LITA	Livestock Training Agency
LR	Likelihood Ratio
LRR	Leucine Rich Region
M	Matrix
MAF	Minor allele frequency
MAGs	Marker Allele Groups
MD	Marek's Disease
MEGA	Molecular Evolution Genetic Analysis
MHC	Major Histocompatibility Complex
ML	Maximum Likelihood
MLD	Minimum Lethal Dose
MUSCLE	Multiple Sequence Alignment By Log-Expectation
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NEB	New England Biolabs
NM-AIST	Nelson Mandela African Institution of Science and Technology
NP	Nucleocapsid
OR	Odds Ratio
P	Phosphoprotein
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction

PCR-LP	Polymerase Chain Reaction Length Polymorphism
PEHPL	Program for Enhancing Health and Productivity of Livestock
PhD	Doctor of Philosophy
PI	Post Infection
RNA	Ribonucleic acid
RT	Real Time
S/P	Sensitivity/Specificity
SNP	Single Nucleotide Polymorphism
SSA	Sub-Saharan Africa
SSR	Simple Sequence Repeats
SUA	Sokoine University of Agriculture
TBE	Tris-Boric Ethylenediaminetetraacetic Acid
TLM	Tanzani Livestock Modernization Initiative
TLR	Toll Like Receptor
UV	Ultraviolet
VNTR	Variable Number of Tandem Repeats

CHAPTER ONE

INTRODUCTION

1.1 Background of the problem

The world human population has increased rapidly in the last five decades, with current world population estimated to be 7.7 billion, and is projected to reach 9.7 billion by 2050 (World Population Review, 2019). In Sub-Saharan Africa (SSA), the human population rose from 186 million to 856 million in the last 60 years and is projected to reach 2.7 billion people by 2060 (World Population Review, 2019). Tanzanian is among the other six SSA countries that will have a significant contribution to the total world population by 2050 (World Population Review, 2019). To meet the nutritional demand of increasing human population in SSA there is a need to increase poultry production.

The world chicken population is estimated at 18 billion (Conan *et al*., 2012), and about 80% of chickens that are raised in Africa are indigenous, local chickens (Conan *et al*., 2012). The majority (60%) of African households raise chickens under backyard production settings where chickens are left to scavenge for their nutritional need (Mapiye *et al*., 2008; Mtileni *et al*., 2009). In Tanzania, 96% of livestock farmers keep local chickens, which supply 94% of poultry meat and eggs in rural areas (Tanzania Ministry of Livestock and Fisheries Development, 2015). Local chickens are well adapted to harsh tropical environmental conditions and survive persistent exposure to endemic infectious diseases (Msoffe *et al*., 2001; Minga *et al*., 2004; Mpenda *et al*., 2019). The growth of chicken is rapid and its farming requires relatively small land size. More importantly, chicken has a high nutritive value from chicken eggs and meat, and serves as a chief source of high-quality meat protein among households in resource-poor countries in SSA (Ahlers *et al*., 2009; Mtileni *et al*., 2009; Martin *et al*., 2015). In African setting, chickens particularly local chickens play a crucial role in socio-cultural functions such as traditional ceremonies and rituals (Mtileni *et al*., 2009; Conan *et al*., 2012). However, local chickens are characterized by low productivity traits like low growth performance and eggs production (Yakubu & Ari, 2018). Efforts have been made to introduce improved chicken genetic resources adapted to harsh tropical environmental conditions, for example, with an introduction of kuroiler and Sasso chickens (Osei-amponsah *et al*., 2010; Yakubu & Ari, 2018). Kuroiler and Sasso chickens also have some genetic background of exotic chicken breeds these have been tested in India and France respectively, but not sure about their adaptability in Africa in terms of climate change and

disease in Africa. Introduction of improved breeds is in agreement with Tanzania Livestock Modernization Initiative (TLMI) of 2015, which among other key priority actions in poultry modernization, is the identification of dual-purpose breeds suitable for local free-range scavenging conditions.

However, the production of chickens is constrained by high mortality rates and morbidities due to infectious diseases (Okeno *et al*., 2012; Marwa *et al*., 2016). Among chicken viral diseases, Newcastle disease (ND) is a major constraint of chicken production under backyard production settings in resources poor countries of SSA (Alexander, 2000; Sonaiya, 2008; Permin & Bisgaard, 2013). A negative single-stranded RNA virus called Newcastle disease virus (NDV) is a causative agent of the disease (Alexander, 2000; Permin & Bisgaard, 2013). Like other viral diseases in animals and birds, Newcastle disease (ND) does not have well-known treatment options; control and prevention depends on proper vaccine administration and appropriate implementation of biosecurity measures to halt spread and transmissions of infections between chicken flocks (Cristalli & Capua, 2007; Conan *et al*., 2012; Mazengia, 2012; Alders, 2014). In backyard systems, the implementation of vaccination and biosecurity measures is a challenge due to farmers' lack of resources to buy vaccines and because local chickens are free ranging (Cristalli & Capua, 2007; Conan *et al*., 2012; Mazengia, 2012).

Selection for chicken resistant to viral infections is a promising approach for control and prevention of Newcastle disease (Bacon *et al.*, 2000). Phenotypic and genotypic individual variations in susceptibility to diseases within and between chicken breeds or ecotypes have been documented (Bacon *et al*., 2000; Msoffe *et al*., 2001; Miller & Taylor, 2016). For example, chicken breeds, which are less susceptible to Marek's disease (MD), have been under development for years (Bacon *et al*., 2000). Breeding for disease-resistant genotypes is a powerful approach that would complement existing disease management options and decrease input cost in chicken production. This explains why there is an urgent need among scientific communities to search for genes conferring disease resistance that can be deployed as disease resistance markers.

Genetic resistance to infectious agents is polygenic and influenced by the interaction of biological and environmental factors (Zekarias *et al*., 2002; Zeleke *et al*., 2005). However, a major gene model that was postulated by Lande (1981) underlines a larger influence of a few genes in genetic variations. The mechanisms of disease resistance are mainly controlled by immune responses, which are comprised of both innate and adaptive immunity (Glass *et al*.,

2012; Kapczynski *et al*., 2013). The major histocompatibility complex (MHC) haplotypes determined by LEI0258 microsatellite marker associated with variation in disease susceptibility and resistance have been reported (Capra *et al*., 2001; Leveque *et al*., 2003; Lwelamira *et al*., 2008; Hunt *et al*., 2010; Kapczynski *et al*., 2013). The myxovirus-resistance (Mx) protein, an interferon (IFN)-induced dynamin-like guanosine triphosphateases (GTPases), is among of the non-MHC genes that play significant antiviral activities (Verhelst *et al*., 2013). The Mx allelic variants have been associated with chicken variability in susceptibility to viral infections (Verhelst *et al*., 2013; Fulton *et al*., 2014). However, molecular mechanisms of chicken variation in susceptibility to diseases, particularly variation in susceptibility to NDV are not fully understood and more remain to be explored in Tanzania. Keeping these facts in view, the present research was aimed to investigate genetic mechanisms of susceptibility to NDV challenge in local Tanzanian chickens and exotics.

1.2 Statement of the problem

Newcastle disease control by vaccination and institution of biosecurity measures is less feasible, in particular under backyard production systems (Alexander, 2001; Sharif *et al*., 2014). Genetic selection of chicken genotypes that are less susceptible to NDV is a promising option. However, the molecular mechanisms of chicken variations in susceptibility to NDV are not well understood and more remain to be explored. The present research aimed to investigate whether there is an association between genetic variants (GVs) of selected candidate genes (LEIO258 and Mx1) and variations in susceptibility to NDV in local Tanzanian chicken and exotics. The information generated from this research is very useful in breeding programs designed to develop chickens that are less susceptible to NDV.

1.3 Rationale of the study

Newcastle disease (ND) is the number one killer, and cause enormous losses in chicken productivity under backyard production (Alexander, 2000; Sonaiya, 2008; Permin & Bisgaard, 2013). More importantly, like other viral infections, ND does not have well-known treatment options. Control and prevention depend on proper vaccine administration and appropriate implementation of biosecurity measures to halt spread and transmissions of infections between chicken flocks (Cristalli & Capua, 2007; Conan *et al*., 2012; Mazengia, 2012; Alders, 2014). However, in backyard systems, the implementation of vaccination and biosecurity measures remain a challenge due to farmers' lack of resources to buy vaccines

and because local chickens are free ranging (Cristalli & Capua, 2007; Conan *et al*., 2012; Mazengia, 2012).

Selection for chickens resistant to viral infections is a promising approach for control and prevention of chicken viral infections (Bacon *et al*., 2000). Phenotypic and genotypic individual variations within and between chicken breeds or ecotypes have been documented (Bacon *et al*., 2000; Msoffe *et al*., 2001; Miller & Taylor, 2016). However, the molecular mechanisms of chicken variations in susceptibility to NDV are not well understood and more remain to be explored. Therefore, the study aimed to investigate whether there is an association between genetic variants (GVs) of selected candidate genes (LEIO258 & Mx1) and variations in susceptibility to NDV in local Tanzanian chicken and exotics.

1.4 Objectives

1.4.1 General objective

To investigate genetic mechanisms of susceptibility to NDV challenge in exotic and local Tanzanian chicken.

1.4.2 Specific objectives

(i) To assess growth performance and antibody responses following ND vaccination in kuroiler, broiler and local Tanzanian chickens.
(ii) To assess variability in susceptibility to virulent NDV challenge within kuroiler, Sasso and local Tanzanian chickens by using chicken embryo model.
(iii) To determine associations between genetic variants of selected candidate genes (Mx1 and LEI0258) and variations in susceptibility to virulent NDV challenge within kuroiler, Sasso and local Tanzanian chickens by using chicken embryo model.

1.5 Research questions

(i) Is there difference in growth performance and antibody responses following ND vaccination in kuroiler, broiler and local Tanzanian chickens?
(ii) What is the level of variability in susceptibility to virulent NDV challenge within kuroiler, Sasso and local Tanzanian chickens by using chicken embryo model?
(iii) Is there an association between genetic variants of selected candidate genes (Mx1 and LEI0258) and variations in susceptibility to virulent NDV challenge within kuroiler, Sasso and local Tanzanian chickens by using chicken embryo model?

1.6 Significance of the Study

Newcastle disease is a major constraint that compromise chicken production among poor rural smallholder farmers in developing countries of SSA. The available disease control and prevention strategies are less effective and feasible under backyard production settings. The study was done to investigate molecular mechanisms of susceptibility to NDV challenge in local Tanzanian chicken and exotics. Results of the study add to the scientific body of knowledge on the molecular basis for chicken variations in susceptibility to virulent Newcastle disease virus. Also, the information generated from this study is very important as it provides a platform for breeding programs designed for development of chicken genotypes that are resistant to virulent NDV.

1.7 Delineation of the study

Initially, chicken susceptibility to NDV was assessed by vaccination of kuroiler, broiler and local Tanzanian chickens with live La Sota NDV vaccine, and body weight gain and antibody responses post-vaccination were used as phenotypes. Although results showed differences between vaccinated and control groups, the results could not give clear cut differences on variation in susceptibility, probably because a less virulent strain of NDV was used and the housing environment might have created some confounding factors as previously described (Schilling *et al*., 2018).

Furthermore, challenging adult chicken with vNDV was difficulty due to lack of facility that could contain vNDV from spillover to surrounding environment. Also, with regard to ethical use of animals in experiment, it was highly unethical of challenging adult chicken with vNDV that could kill all birds. Therefore, in subsequent experiment virulent NDV and

chicken embryo model were used to investigate chicken variation in susceptibility to NDV, and survival time post-challenge was used as a phenotype.

Also, findings from the present study demonstrated comparable antibody titre against NDV in kuroilers and local chicken. It should be noted that, only local chickens from different parts of Arusha region were involved, and it remain to be demonstrated whether similar findings can be generated if local chicken from different parts of Tanzania are involved.

CHAPTER TWO

LITERATURE REVIEW

2.1 Local and exotic chickens

Local chickens are chickens that are reared under backyard production systems (Sonaiya, 2008; Conan *et al*., 2012). Various names are used depending on the country of origin to refer to local chicken (Table 1). In this thesis, the name 'local chicken' refers to indigenous chicken genotypes adapted to harsh tropical environmental conditions. The main characteristic of local chickens under backyard production system is free-range movement of birds, which allow them to scavenge for nutritional requirements (Kitalyi, 1998; Guèye, 2000; Mtileni *et al*., 2009). Local chicken production is cost-effective and very appropriate among resources poor rural households of SSA, as it does not necessarily require feed supplementation. Local chicken production systems has been reviewed in detail elsewhere (Guèye, 2000; Mapiye *et al*., 2008).

Nevertheless, local chickens are characterized by low productivity traits like low growth performance and eggs production (Yakubu & Ari, 2018). To mitigate local chicken low productivity challenge, there is a concerted effort to introduce improved dual-purpose chicken genetic resources adapted to harsh tropical environmental conditions, for example, the kuroiler and Sasso chickens (Osei-amponsah *et al*., 2010; Yakubu & Ari, 2018). The dual-purpose exotic breeds like kuroilers and Sasso are characterized by rapid body weight gain and a high number of egg production as compared to local chickens. Introduction of improved breeds is in an agreement with Tanzania Livestock Modernization Initiative (TLMI) of 2015, which among other key priority actions in poultry modernization is the identification and introduction of dual-purpose breeds suitable for local free-range scavenging conditions of Tanzania.

A new hybrid chicken called the kuroiler was introduced to Africa from India (Sharma *et al*., 2015; Fleming *et al*., 2016; Yakubu & Ari, 2018). The kuroiler is dual-purpose scavenger chicken raised for egg and meat production (Dessie & Getachew, 2016). Like local chickens, kuroilers can thrive under harsh tropical environmental conditions, and they can scavenge for nutrition needs just like local chickens (Isenberg, 2008; Dessie & Getachew, 2016). The breed outperforms local chickens in terms of meat and egg production (Isenberg, 2008; Yakubu & Ari, 2018). In a pilot study conducted in Uganda, kuroilers and local Ugandan chickens kept under the same scavenging settings, and the kuroilers had higher production

performance compared to local Ugandan chickens (Sharma *et al*., 2015).

At 25 weeks of age, the average body weight of the male kuroiler chickens was 2.6 kg, compared to 1.6 kg for the male local Ugandan chicken (Sharma *et al*., 2015). Additionally, at 6-weeks of age, kuroilers had higher average body weight than the Sasso chicken which originated from France and the Fulani, an indigenous chicken from Nigeria (Yakubu & Ari, 2018). Furthermore, the kuroiler chicken produced 4-5 times more eggs compared to the Desi, a local Indian chicken breed (Isenberg, 2008). Although not supported by empirical studies, kuroilers are said to be resistant to infectious diseases (Sharma *et al*., 2015; Fleming *et al*., 2016). Thus, increased production of kuroiler chicken may have a significant impact on improving the quality of livelihood for resource-poor rural households.

Table 1: **Summary of production dynamic studies for local chickens in Africa**

Study	Country	Name*	Production system	Average flock size	Reason of keeping chickens	Constraints
Yousif *et al*. (2015)	Sudan	Native chicken,	Extensive /backyard	25	Meat provision and cash generating	Infectious diseases, predators,
Marwa *et al*. (2016)	Tanzania	Rural chicken	Extensive /backyard	5	Food, sale (source of income)	Poor nutrition, Infectious diseases, predation
Okeno *et al*. (2012)	Kenya	Indigenous chicken	Small-scale free range	22	Food and cash income	Infectious diseases, Poor nutrition
Getu and Birhan, (2014)	Ethiopia	Indigenous chicken	Extensive	16	Food, source of income	Infectious diseases and predation
Gondwe and Wollny, (2007)	Malawi	Scavenging chicken	Scavenging	13	Food, socio-cultural functions,	-

Legend: *various names, which are synonymously, used referring to local chickens

Like kuroiler, Sasso is a dual-purpose French chicken breed, which is raised for meat and eggs production. The breed has been introduced in Africa through the Africa Chicken Genetic Gains Program (ACGG). The breed thrives well in harsh tropical environmental conditions and is free ranging chicken with the ability of scavenging. The breed is well known for fast growth performance and high eggs production (Osei-amponsah *et al*., 2010). Therefore, because of good productivity performance of these exotic chicken breeds (kuroilers and Sasso), there is a need to evaluate their genetic ability to respond to viral infections particularly infection with the Newcastle disease virus.

2.2 Newcastle disease

A major constraint of chicken production under backyard settings is diseases, in particular, infectious diseases (Table 1). Viral infections that rank as most important to smallholder farmers in developing countries of SSA are Newcastle disease (Kitalyi, 1998; Awuni, 2002; Gondwe & Wollny, 2007).

Newcastle disease is the principal constraint to chicken production in backyard production systems (Alexander *et al*., 2004). The disease is caused by the NDV, an avian Paramyxovirus serotype 1 (Alexander, 2000; Alexander *et al*., 2004). The mortality of NDV infection in the naïve susceptible flock can reach up to 100% (Samuel *et al*., 2013). The majority (80%) of losses in local chicken production and about half of the early chick mortalities (chicks from hatch to six weeks) caused by ND. The disease has an enormous economic impact on the global poultry industry due to losses caused by high morbidity and mortality rates (Alexander, 2000, 2001; Pedersen *et al*., 2004). For example, to control the spread of the disease, the 2002 exotic ND outbreak in California led to the culling of 3.5 million birds (Pedersen *et al*., 2004).

2.3 Newcastle disease virus

The NDV is the member of the genus *Avulavirus*, sub-family Paramyxovirinae, family Paramyxoviridae, and the order Mononegavirales (Pedersen *et al*., 2004; Samuel *et al*., 2013). The NDV is an enveloped single-stranded negative-sense RNA genome (Alexander, 2001; Heiden *et al*., 2014). The genome of NDV is approximately 16 kilobase (kb) in length and consists of six genes (Alexander, 2001; Pedersen *et al*., 2004; Samuel *et al*., 2013). The genes encode seven viral proteins including the nucleocapsid (NP), phosphoprotein (P), matrix (M), fusion (F), hemagglutinin-neuraminidase (HN), and RNA-directed RNA polymerase proteins

(L) (Aldous *et al*., 2003; Xiao *et al*., 2012; Farooq *et al*., 2014). There are three types of NDV based on pathogenicity: lentogenic, mesogenic, and velogenic (Aldous *et al*., 2003; Xiao *et al*., 2012; Farooq *et al*., 2014).

The Office International des Epizooties (OIE) guideline requires characterization of NDV virulence with an intra-cerebral pathogenicity index (ICPI) of ≥ 0.7 in day-old chicks or molecular determination of the presence of multiple basic amino acids at the F protein cleavage site (Alexander, 2000; Aldous *et al*., 2003; Farooq *et al*., 2014). The virulent NDV strains have the high content of basic amino acids residues at the F0 protein cleavage site (Glickman *et al*., 1988); either lysine (K) or arginine (R) at residue 112 to 113 and 115 or 116 and a phenylalanine residue 117 (Glickman *et al*., 1988). The multiple basic amino acids allow cleavage of the F0 protein into two subunits (F1 and F2) by a variety of host proteases found in most tissues (Toyoda *et al*., 1989; Morrison, 2003). The velogenic and mesogenic NDV strains have a 112R/K-R-Q-R/K-RF117 F protein cleavage site motif; whereas, lentogenic NDV strains have a 112G/E-K/R-Q-/E-RL117 motif (Glickman *et al*., 1988). The presence of multiple basic amino acids at the F0 cleavage sequence is an essential criterion for confirming the virulence of NDV (Aldous *et al*., 2003).

Novel virulent NDV strains have been reported in chicken flocks in Africa continent (Kim *et al*., 2012; Samuel *et al*., 2013). The evolution and emergence of new virulent NDV genotypes may explain the reported cases of vaccine failures (Snoeck *et al*., 2009; Kim *et al*., 2012; Samuel *et al*., 2013). Along with the effort to develop antigenic-matched ND vaccines to improve the efficacy of the current ND vaccines, selection of chicken resistant to the circulating virulent NDV strains is a promising alternative strategy.

2.4 Newcastle disease control options

Under backyard production settings, the prevention and control of ND is a challenge. The control and prevention of ND largely depend on vaccination and institution of appropriate biosecurity measures (Alexander, 2001; Sharif *et al*., 2014). Like other viral infections in farmed animals and birds, ND lack treatment options.

Vaccination adoption significantly decreases chicken mortalities from NDV infections (Van der Goot *et al*., 2005; Sharif *et al*., 2014). For example, in a community-based ND vaccination program conducted in Tanzania, its effectiveness was almost 70% (Msoffe *et al*., 2010). Under experimental settings, ND vaccination can result in 100% of flock protection to

the disease (Ali *et al*., 2014). However, control of ND by vaccination remains a challenge, particularly in rural areas due to lack of vaccination programs and improper vaccination attributed to limited veterinary extension services in rural areas (Blackie, 2014). Limited financial resources among poor rural households to purchase vaccines and lack of storage facilities, like unavailability of the cold chain for ND vaccines storage, are significant constraints facing vaccination adoption in backyard production systems (Alexander, 2001; Marangon & Busani, 2007). Furthermore, ND control by vaccination has limited ability to prevent the spread and transmission of viruses to uninfected chickens (Marangon & Busani, 2007). The vaccination aims to prevent clinical disease (Marangon & Busani, 2007), and vaccinated birds can shed viruses in their secretions such as in feces, tears and mucosal thereby spreading infections in the surrounding area (Davison & Nair, 2005).

Another strategy is bio-security, which is designed to prevent the spread of infectious agents within and between flocks. Biosecurity has three main components: isolation, traffic control, and sanitation (Conan *et al*., 2012). The main characteristic of isolation is the confinement of birds within a controlled environment (Conan *et al*., 2012; Pollock *et al*., 2012). Traffic control is designed to limit movement within and between flocks. Sanitation deals with disinfection of materials, people, and equipment entering and leaving the farm, and cleanliness of farm caretakers (Pollock *et al*., 2012). Biosecurity is an essential means of preventing infections; however, not much information is available to support its feasibility in backyard production systems (Fasina *et al*., 2012). A systematic literature review by Conan *et al*. (2012), found that most of the biosecurity measures devised under intensive production systems are not feasible or effective under backyard chicken production systems. Given the challenges of implementing vaccination and biosecurity measures in backyard systems, genetic selection of chicken's resistant to NDV could be a more robust and cost-effective solution to preventing and controlling ND.

2.5 Chicken genetic diversity

Genetic diversity is derived from genes, segments of DNA that contain essential information for all life on earth (Springbett *et al*., 2003). The concept of genetic variation derives from the possibility that individuals in a given population may carry polymorphic (different) DNA sequences of a given genomic region (Springbett *et al*., 2003). Therefore, genetic diversity can be defined as varieties of genes within a species (Springbett *et al*., 2003). The genetic diversity is at three levels: species, population, and individual (Jovanović *et al*., 2009).

Within a population of a given species, individuals may have unique genetic composition resulting in genetic variability among members of the same species leading to population sub-structuring (Rao & Hodgkin, 2002). Individual genomic differences in population are a determinant of genetic diversity of a given population (Rao & Hodgkin, 2002; Springbett *et al*., 2003; Keambou *et al*., 2014).

Genotypic diversity analysis of microsatellite markers has been extensively used to evaluate the genetic diversity and population structure in chickens (Muchadeyi *et al*., 2007; Mwacharo *et al.,* 2007; Mtileni *et al*., 2011). Based on the mean number of alleles (MNA) and heterozygosity values, reports indicate that chickens from different parts of the world are highly genetically diverse as compared to pure commercial breeds (Table 2). When compared to local chickens from other parts of the world, local African chickens have demonstrated higher genetic diversity (Table 2).

Based on population structure indices, reports indicate that local chicken populations are genetically closely related (Mwacharo *et al*., 2007; Chen *et al*., 2008; Mtileni *et al*., 2011). The genetic variation of local chickens is largely accounted for by the within ecotype variations. For example, 96.8% of the total variation among Zimbabwean chickens attributed to within ecotype variations (Reed & Frankham, 2003). Similar findings observed among local chicken ecotypes of Tanzania (Lyimo *et al*., 2013), Kenya (Mwacharo *et al*., 2007), Egypt (Eltanany *et al*., 2011), Sudan (Hasballa, 2008), Ethiopia (Goraga *et al*., 2012), South Africa (Mtileni *et al*., 2011) and Algeria (Mahammi *et al*., 2016). The available information suggests little differentiation among outbred chicken populations.

Table 2: **Genetic diversity and population structure statistics estimated in local and commercial chickens**

Country	Chicken type	MNA[1]	Ho[2]	Fst[3]	Reference
Ethiopia	Local chicken	11	0.5	0.12	Bekerie *et al*. (2015)
Tanzania	Local chicken	5.7	0.62	0.05	Lyimo *et al*. (2013)
South Africa	Local chicken	6.6	0.6-	0.01	Mtileni *et al*. (2011)
	Conserved local chicken*	4.7	0.5	0.16	
Algeria	Local chicken	7.1	0.5	-	Mahammi *et al*. (2016)
	Commercial	3.9	0.2	-	
Sudan	Local chicken	5.3	0.5	0.03	Yousif *et al*. (2013)
	Commercial	3.2	0.4	0.32	
Ghana	Local chicken	6.6	0.6	0.01	Osei-amponsah *et al*. (2010)
	Commercial	6.0	0.5	0.29	
Egypt	Local chicken	6.9	0.6	0.08	Ramadan *et al*. (2012)
Britain	British local chicken	3.6	0.4	0.25	Wilkinson *et al*. (2012)
Sweden	Swedish local chicken	4.7	0.3	0.44	Abebe *et al*. (2015)

Legend: [1]Mean number of alleles per locus; [2]average observed heterozygosity; [3]population differentiation index; *the local chicken under conservation program characterized by limited movement leading to inbreeding

2.6 Genetic diversity and disease susceptibility variability

Genetic diversity contributes to population fitness in response to a changing environment (Zhu *et al*., 2000; Reed & Frankham, 2003; Shapiro, 2016). Population genetic variations allow individual adaptation to the changing environment like adaptations to endemic infectious diseases and climate change (Räikkönen *et al*., 2006; Allentoft & O'Brien, 2010). The correlation between genetic diversity and population fitness was demonstrated using molecular quantitative genetics data (Reed & Frankham, 2003). Springbett *et al*. (2003) demonstrated using stochastic models that a heterogeneous population is less likely to suffer from catastrophic disease epidemics as compared to homogeneous population (Springbett *et al*., 2003). In contrast, the loss of genetic diversity has been associated with species extinction (Zhu *et al*., 2000; Reed & Frankham, 2003; Shapiro, 2016).

Disease resistance is defined as the ability of the host to resist infections (Jovanović *et al*., 2009). For the host to resist infection, the infectious agent should be cleared before getting into the host cell. The resistant host should prevent pathogen attachment and entry into the host cell (Jovanović *et al*., 2009). In contrast, disease tolerance is the ability of the host to suffer minimal adverse effects following infection (Råberg *et al*., 2007; Jovanović *et al*., 2009).

At the molecular level, mechanisms of disease resistance are complex and not fully understood. Genetic resistance to infectious agents is polygenic and influenced by the interaction of biological and environmental factors (Zekarias *et al*., 2002; Zeleke *et al*., 2005). The mechanisms of disease resistance are mainly controlled by immune responses, which are comprised of both innate and adaptive immunity (Glass *et al*., 2012; Kapczynski *et al*., 2013). The innate immune response is the first line of defense, which clears pathogens in the very early stages of infection and initiates adaptive immune responses (Kapczynski *et al*., 2013; Iwasaki & Medzhitov, 2015).

The major histocompatibility complex (MHC) haplotypes are associated with variation in disease susceptibility and resistance (Capra *et al*., 2001; Leveque et al., 2003; Hunt *et al*., 2010; Kapczynski *et al*., 2013). The MHC molecules play a significant role in antigen processing and presentation (Janeway *et al*., 2001). The MHC is mainly involved in antigen processing and presentation of all possible antigens due to the presence of multiple variants of each gene within the population (Janeway *et al*., 2001). The MHC genotype restricts the

antigen recognition by T-cells. The defect in the MHC molecule may prevent the antigen recognition by specific T-cells. Therefore, antigen specificity of T-cell is controlled by MHC molecules (Janeway *et al*., 2001).

Reports indicate that chicken variations in susceptibility to infectious diseases are linked with the MHC haplotypes (Schat *et al*., 1994; Goto *et al*., 2009). For instance, antibody responses to NDV are associated with two LEI0258 microsatellite alleles, 205 bp and 307 bp, in Tanzanian chicken ecotypes (Lwelamira *et al*., 2008). The allelic variant 205 bp was shown to be positively associated with the elevated level of antibody responses to NDV vaccine; whereas, allelic variant 307 bp was negatively associated with the same trait (Lwelamira *et al.*, 2008). Also, chicken with the same genetic composition may respond differently when exposed to different infectious agents. Chicken populations with similar MHC haplotype (B1B1) vary on antibody responses to *S. pullorum* and susceptibility to MDV infection (Pevzner *et al*., 1981). Interestingly, chickens with high antibody responses to *S. pullorum* antigen had high mortality rate to MDV challenge whereas chickens with low antibody responses to *S. pullorum* antigen had low mortality rates in response to MDV challenge (Pevzner *et al*., 1981). The association of the MHC variants with chicken variations in susceptibility to disease is reviewed elsewhere (Miller & Taylor, 2016).

The toll-like receptor genes signaling and interferon-signaling feature explain a significant role played by non-MHC genes in chicken variations in susceptibility to diseases (Ruan & Zheng, 2011; Haunshi & Cheng, 2014). Toll-like receptors (TLRs) constitute a group of pathogen-associated molecular patterns (PPRs) that play a crucial role in infectious agents recognition and induction of innate immune response (Majewska & Szczepanik, 2006). Although the role of TLRs in chicken variations in susceptibility to diseases is not fully understood, reports indicate that TLRs allelic variants are associated with variability in chicken resistance to diseases (Table 3). Specifically, two non-conservative mutations within the Leucine-rich region (LRR) domain (Tyr383His and Gln611Arg) of TLR4 are associated with susceptibility to *Salmonella* infection in two lines of chickens (line C and 72).

The Mx protein, an interferon (IFN)-induced dynamin-like GTPases, is among non-MHC genes that play significant antiviral activities (Verhelst *et al*., 2013). The Mx allelic variants have been associated with chicken variability in susceptibility to viral infections (Verhelst *et al*., 2013; Fulton *et al*., 2014). The Mx gene genotypes (genotype AA and GG) among Indonesian chicken, Tolaki, had significant differences in antiviral activities. Genotype AA

had a higher antiviral activity of 50% compared to genotype GG, which had the antiviral activity of 10% (Pagala *et al*., 2013).

Table 3: Genes/variants associated with disease resistance/susceptibility in chickens

Disease	Gene	Role	Reference
Salmonellosis	TLR4	Two non-conservative change in LRR domain associated to chicken susceptibility to salmonellosis	Leveque *et al.* (2003)
Salmonellosis	NRPMP1	Single SNP A101991G associated with chicken resistance to salmonellosis	He et *al*. (2013)
Marek'sdisease	GH1	Conferred chicken resistance to MF	Liu *et al*. (2011)
Marek's disease	CHTF18	Indle mutation with an additional of 7 nucleotides associated with chicken resistance to MD	Kaya *et al*. (2016)
Lympoid leukosis	LDLR	Cysteine-to-tryptophan change in low-density lipoprotein receptor (LDLR) for Avian leukosis viruses reduces binding affinity of the virus.	Elleder *et al*. (2004)
Influenza	Mx gene	Amino acid substitution of Mx protein at position 631 (Ser to Asn) enhance antiviral activities in chicken	Ko *et al*. (2002)
Coccidiosis	LEI 0071	The LEI 0071 is associated with reduction of parasite growth (*Eimeria.maxima*) in chicken.	Lillehoj *et al*. (2008)

2.7 Molecular markers

A genetic or molecular marker can be defined as a gene or DNA sequence that is associated with a particular gene or trait and its location on a chromosome is known (Al-Samarai & Al-Kazaz, 2015). It is the variation with a genome that arises due to mutation and other alterations within a genome, which can be observed using molecular techniques. There are three main types of variation at DNA level from molecular mechanism point of view: Single nucleotide change or polymorphism (SNP); insertions or deletions (Indels) and VNTR for variations in the number of tandem repeats (Vignal *et al*., 2002; Al-Samarai & Al-Kazaz, 2015). Molecular markers are very useful in animal genetic studies like marker-assisted selection strategies, parentage testing, species identification, and population genetic studies as they uncover polymorphism at the DNA level (Vignal *et al*., 2002; Al-Samarai & Al-Kazaz, 2015). Molecular markers are useful in "Smart breeding" where molecular markers are used in breeding strategies. The SNP and microsatellite markers are reviewed in detail here below.

2.7.1 Single nucleotide polymorphism

As mention earlier, the SNP (Single nucleotide polymorphism) marker is a single nucleotide alteration in a DNA sequence, which is usually bi-allelic (Skevaki *et al*., 2015). For a single change in base at DNA sequence to be considered SNP, a minor allele should have a frequency of 1% (Vignal *et al*., 2002; Al-Samarai & Al-Kazaz, 2015). Single nucleotide change or polymorphisms are highly abundant and genetically stable molecular marker, which are distributed throughout the genome. Single nucleotide change or polymorphisms are found in both coding and non-coding regions of the genome (Vignal *et al*., 2002; Skevaki *et al*., 2015). The frequency of SNPs is 5 times higher in the chicken genome as compared to humans. Single nucleotide change or polymorphisms are very useful in smart animal breeding and genetic studies. They are used in population genetic diversity studies and in association studies mapping of genes that are in linkage disequilibrium with complex traits (He *et al*., 2013; Fulton *et al*., 2014; Skevaki *et al*., 2015).

2.7.2 Microsatellites

Microsatellites are defined as simple sequence repeated (SSR) loci, and is also known as variable number of tandem repeats (VNTRs) and simple sequence length polymorphisms (SSLSPs) (Al-Samarai & Al-Kazaz, 2015). The SSR are found in entire genome of most eukaryotes. SSRs are co-dominant multi-allelic and highly polymorphic at a given locus. The

tandem number of repeats determines the SSRs allelic sizes. Microsatellites are easy to detect and is reliable measure of genetic diversity. Microsatellite have been used to build high-density genetic maps, which are used to locate desired traits including disease resistance traits (He *et al*., 2013; Skevaki *et al*., 2015).

2.8 LEI0258 microsatellite marker

The LEI0258 microsatellite marker is a tandem repeat genetic marker, which is physically located within B-F/B-L region of chicken MHC-B (Fig. 1). The LEI0258 is the most highly polymorphic genetic marker within the MHC-B region, and more importantly is associated with serologically identified chicken MHC haplotypes (Fulton *et al*., 2006). Allelic variants of LEI0258 marker have been used for genetic diversity studies in chicken populations, and reflecting MHC variability in chicken populations (Fulton *et al*., 2006).

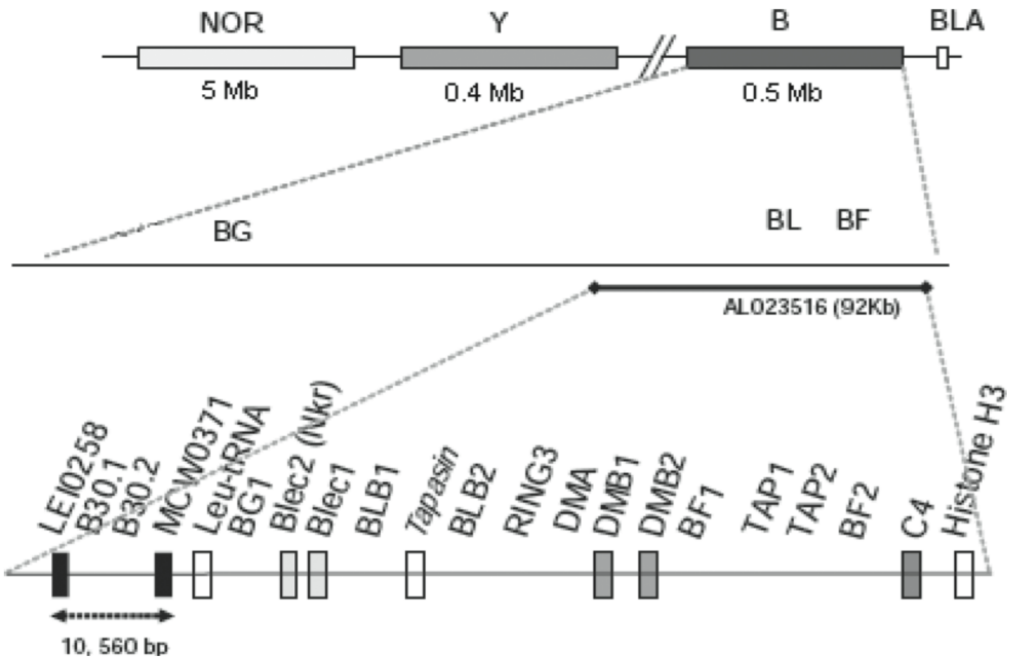

Figure 1: The chicken Major Histocompatibility Complex map (modified from Kaufman *et al.* (1999) with updated information from Delany *et al.* (2009) and Solinhac *et al.* (2010) showing the location of marker LEI0258. Cosmid cluster 1 sequenced genes are indicated.

The chicken Major histocompatibility Complex-B (MHC-B) is widely studied for its crucial role in disease resistance, susceptibility, and variability in response to vaccines. The role of chicken MHC-B in genetic resistance to viral diseases has been documented, including Marek's disease (Martin *et al*., 1989), avian leucosis (Yoo & Sheldon, 1992) and avian influenza virus (Hunt *et al*., 2010). Chicken variability in resistance and susceptibility to

diseases have been linked with MHC haplotypes identified by using LEI0258 marker (Schat *et al*., 1994; Goto *et al*., 2009). Lwelamira *et al*. (2008), demonstrated an association between chicken antibody responses against NDV and 2 MHC haplotypes (alleles 205 bp and 307 bp) determined by LEI0258 microsatellite marker. One allele (205) was positively associated with the trait, while another allele (307) had opposite effect on the same trait (Lwelamira *et al*., 2008). Chicken MHC haplotypes, which have been involved in association studies on chicken variability in resistance and susceptibility to infectious diseases, most are determined by allelic variants of LEI0258 microsatellite marker (McConnell *et al*., 1999; Lima-Rosa *et al*., 2005; Fulton *et al*., 2006).

Various approaches have been deployed in genotyping the LEI0258 marker. Most common techniques are genotyping by PCR followed by electrophoresis and genotyping by sequencing (Fulton *et al*., 2006; Lwelamira *et al*., 2008; Han *et al*., 2013). The later, apart from determining allelic size of the marker, it provides additional information regarding repetition of tandem repeats (12 and 13 bp repeats), and polymorphisms in upstream and downstream of flanking regions (Fulton *et al*., 2006; Han *et al*., 2013).

2.9 Chicken Mx gene

The myxovirus-resistance (Mx) genes are found in a wide range of living organisms including chicken (Verhelst *et al*., 2013). Mx proteins are interferon (IFN) induced GTPase enzymes with antiviral functions, particularly plays a significant role in the inhibition of negative-stranded RNA viruses (Verhelst *et al*., 2013). Binding of type I or III IFNs to receptors trigger expression of IFN-stimulated genes, thereby induces an antiviral state within a cell. Therefore, expression of Mx genes largely depend on the activation of type I or III IFNs (Hug *et al*., 1988; Aebi *et al*., 1989; Simon *et al*., 1991).

The genomic size of chicken Mx gene is about 21 kb (Fig. 2) with the coding sequences of about 2118 bp, which encodes Mx protein with 705 amino acids (Li *et al*., 2007). Like other IFN-response genes, chicken Mx gene contains a sequence element in their promoters that serve as inducible enhancers. Chicken Mx gene promoter contain a motif 5' AGGTTTCTTTCCT3' or its reverse complement (Schumacher *et al*., 1994; Yin *et al*., 2010), which is an integral part of IFN-stimulated response element (ISRE). It has been documented that the ISRE motif has a crucial role to IFN inducibility of chicken Mx gene (Schumacher *et al*., 1994).

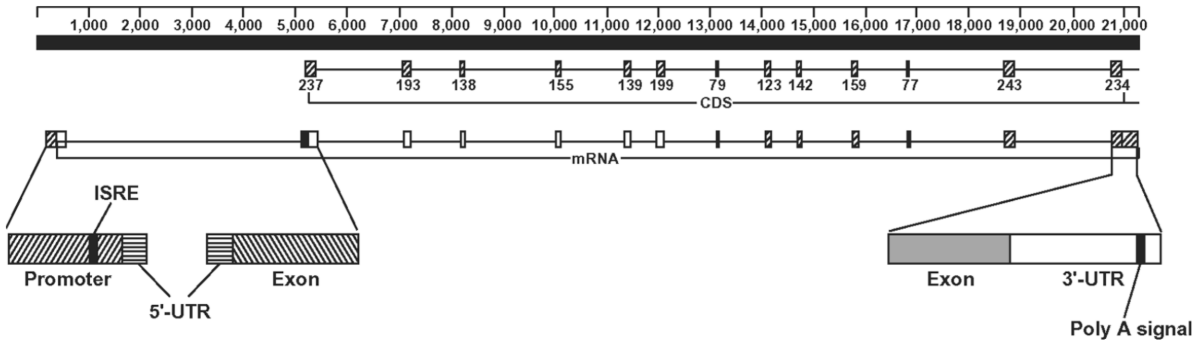

Figure 2: Genomic organization of Mx1 gene in chicken; Derived from Li *et al.* (2007)

Multiple allelic variants of chicken Mx gene have been reported in different populations of chicken throughout the world (Li *et al*., 2007; Wang *et al*., 2012; Fulton *et al*., 2014). For example, Li *et al*. (2007), reported a total of 24 single nucleotide polymorphisms (SNPs) after comparison of four chicken sequences. The highest nucleotide diversity (π value; 0.01003) was in chicken Mx gene promoter where a total of six SNPs were found (Li *et al*., 2007). A similar finding was observed when nine elite egg-layer type line were sequenced where a total of 6 SNPs out of 36 SNPs that were reported were found in chicken Mx gene promoter (Fulton *et al*., 2014).

Most of the reports have focused on chicken Mx gene G2032A (S631) SNP, which has been linked to antiviral activities (Ko *et al*., 2002, 2004; Pagala *et al*., 2013). However, for example, other reports found no evidence of association between chicken Mx gene G2032A polymorphisms and resistance to influenza virus (Benfield *et al*., 2010; Wang *et al*., 2012). These conflicting reports suggests that the role that may be played by chicken Mx in antiviral activity may involve other variants taken into consideration the number of polymorphic sites reported for the chicken Mx gene. Therefore, further investigation on the role of chicken Mx gene polymorphisms and association with resistance to rival infection is intriguing.

CHAPTER THREE

MATERIALS AND METHODS

3.1 Growth performance and antibody responses following ND vaccination in kuroiler, broiler and local Tanzanian chicken

In this first part of experiment, kuroiler, broiler and local Tanzanian chickens were vaccinated with live La Sota ND vaccine and body weight gain and antibody responses were recorded to evaluate variations in chicken susceptibility to NDV.

3.1.1 Chicken population and husbandry

Three chicken breeds (local Tanzanian chicken, kuroilers and broilers), which were raised under the same environment and management condition were involved in the present study. Local Tanzanian chicken and kuroiler eggs were obtained from Urio Cross and Pure Breeding LTD, a local farm in Tanzania (Tengeru, Arusha, Tanzania). Eggs were incubated to hatch at the Nelson Mandela African Institution of Science and Technology (NM-AIST) Laboratory egg incubators. The incubation conditions were 37.9^{o}C temperature and 55% humidity. The eggs were candled at 10 and 18 days of incubation to detect infertile eggs and dead embryos. On the other hand, because of difficulty of obtaining broiler chicken eggs, instead, day-old broiler chicks were obtained from commercial poultry company in Arusha, Tanzania (Tanzania Poultry Farm, Usa River, Arusha Tanzania). Therefore, the experiment started with chicks of the same age, and was housed at Livestock Training Agency-Tengeru campus (LITA-Tengeru). The broiler starter feed (0 – 28 days) and finisher (29 – 50 days) were provided *ad libitum*. Light was provided throughout the experiment and was also used for room temperature adjustment during brooding. The feeds manufactured by commercial company (Harsho Milling Co. Ltd, Moshi Kilimanjaro, Tanzania) were used throughout the experiment.

3.1.2 Experimental design

Day-old chicks were kept in brooding chicken facility at LITA-Tengeru and kept for four weeks to allow maternal antibodies to wane (Jalil *et al*., 2010). Birds at three weeks of age before challenge were wing-tagged and were randomly distributed between the control group A (87 birds) and the challenge group B (271 birds). Birds in control and challenge groups

were kept in separate houses. Group B birds were randomly allocated in three replicates for each chicken type, and birds in each replicate were randomly kept in separate pen of 1.5 m^2. The experiment involved a total of 358 chickens, which were raised for seven weeks (Table 4). Chicken in control group was aimed to monitor possibility of NDV contamination from the environment. Therefore, number of birds in control group was put to minimal, which is in an agreement with ethical use of experimental animals.

Table 4: Description of chicken that were involved in the experiment

Breed	N[1]	Sex		Treatment	
		Male	Female	Control	Challenged
Broiler	121	57	64	28	93
Kuroiler	127	62	65	33	94
Local	110	39	71	26	84

Legend: [1]Total number of chickens involved in the study for each breed

3.1.3 Growth performance assessment

Body weights (BW) in grams (g) were recorded weekly using analytical balance to assess chicken growth performance. Weekly average BW was calculated for the duration of the experiment (7 weeks). The effect of vaccination on the chicken growth performance was evaluated by calculating the mean body weight gain (BWG). The BWG was obtained by taking the body weight difference between week seven (BW, 21 days' post vaccination) and Week four (BW pre-vaccination).

3.1.4 Virus and vaccination protocol

Newcastle disease virus live vaccine (Vir 116, Freeze Dried, LaSota Strain, Biovac, Akiva, Israel) was dissolved in 40 µl sterile normal saline in accordance with the manufacturer instructions to reconstitute to virus titer of $10^{6.5}$ egg infectious dose fifty ($10^{6.5}$ EID50). The vaccine was stored at 4^oC until use as recommended by the manufacturer. The viability of the reconstituted virus was confirmed by inoculating 10-day-old embryonated chicken eggs with 0.1 ml of the reconstituted virus suspension. At four weeks of age birds in the challenge group were inoculated via an ocular route with 100 µl of the virus suspension ($10^{6.5}$ EID50), 50 µl into each eye using a micropipette. Likewise, birds in control group were given 100 µl of phosphate buffered saline (PBS) as mock infection via the same route.

3.1.5 Blood collection and processing

Initially, blood samples were collected from chickens that were selected randomly before vaccination to evaluate whether there was a prior expose of chickens to NDV. Subsequent blood collection was conducted on days 10 and 21 post vaccination. The blood samples were drawn from wing vein into sterile micro-tubes. Blood samples were properly labeled by using the same wing tag number assigned to each bird. Blood samples were left at room 4^0C for overnight to allow coagulation, which was then followed by centrifugation at 3000 rpm to harvest sera. Sera samples were transferred into clean sterile micro-tubes for storage at -20^{o}C until use.

3.1.6 Assessment of antibody levels post-vaccination

Antibody titers were quantified using NDV enzyme linked immunosorbent assay (ELISA) antibody test kit (BioCheck Ltd., Hounslow, London, UK). Sera samples were tested in duplicate in accordance with the manufacturer instructions. Sample optical density was determined spectrophotometrically using 96 wells microliter plate reader (SYNERGY|^{HTX} multi-mode reader, BioTek Instruments Inc, Winooski, VT, USA) at 405 nm wavelengths. The calculation and interpretation of ELISA results was in accordance with the manufacturer guidelines. Briefly, the sensitivity/specificity (S/P) ratio was calculated by using the formula: (mean of test sample−mean of negative control)/(mean of positive control−mean of negative control), and then at 1:500 dilution, the Log10 titer =1.0*Log (S/P) +3.52. Serum sample with S/P value ≥ 0.35 or titer value ≥ 1159 was considered positive for antibodies against NDV.

3.2 Assessment of survival variability upon challenge with virulent NDV in kuroiler, Sasso and local Tanzanian chicken embryos

In this second part of experiment, kuroiler, Sasso and local Tanzanian chicken embryos were challenged with virulent NDV, and the survival time post-challenge was used to assess chicken variations in susceptibility to ND. Kuroiler and Sasso chicken were included in the study because of their good productivity performance, and taken into consideration recent introduction of kuroiler and Sasso in Tanzania. There is a need to evaluate their genetic ability to respond to viral infections particularly infection with the NDV.

3.2.1 Biological and molecular characterization of virulent NDV field isolate

(i) Virulent NDV field isolate

A virulent NDV field isolate from live bird market in Morogoro, Tanzania was kindly provided by Sokoine University of Agriculture (SUA). The isolate was shipped to NM-AIST, and was characterized to confirm its virulence before challenging the chicken embryos (CEs). Characterization was performed as it was previously described (Alexander & Chettle, 1977; Grimes, 2002; Wise *et al.,* 2004; Kim *et al*., 2007). The virus was then titrated to a working titer of minimum lethal dose (MLD) of 10^3 / 0.1 ml of virus suspension and the viral suspension was stored at -80^{o}C until use.

(ii) Virus propagation, titration and pathogenicity tests

Initially, the virus was propagated by inoculating five 10-day-old embryonated chicken eggs with 0.1ml of virus homogenate into the allantoic cavity. Negative control, embryonated eggs were inoculated with a mock 0.1ml of phosphate buffered saline (PBS) via the same route. Both infected and negative control eggs were incubated at 37.9^{o}C, and were first candled 24 hours post-infection (pi) for detection of early embryos death, a sign of bacterial contamination. Subsequently, CEs were candled after every 12 hours for a total of 96 hours. Dead embryos were chilled at 4^{o}C overnight before collection of allantoic fluid for hemagglutination (HA) and hemagglutination inhibition (HI) tests.

Chicken red blood cells (cRBCs) were prepared following protocol described in the previous study (Grimes, 2002). Briefly, whole blood (3 ml) was drawn from the wing vein using 2.5 ml syringe into a tube containing 3 ml of Alsever's solution (Bukantz *et al*., 1946). Then, the blood was washed three times with PBS. The HA was performed using 0.5% CRBCs in 96-well V bottomed micro-titration plates as previously described (Alexander & Chettle, 1977). The HI test was conducted using NDV antiserum to confirm the presence of NDV. Positive allantoic fluids were pooled, aliquoted, and stored at -80^{o}C.

The allantoic fluid was tenfold (10^{-1} to 10^{-9}) serially diluted in sterile PBS. The 0.1 ml of allantoic fluid inoculated in the allantoic cavity of 10-day-old embryonated egg, and a total of five embryos were inoculated with each dilution. Inoculated eggs were incubated at 37^{o}C, and were first candled 24 hours pi to detect early dead embryos. Then, eggs were subsequently examined twice every day for seven days, and embryo time of death was

recorded. The minimum lethal dose (MLD), the highest dilution that killed all inoculated embryos, and mean death time (MDT), defined as the average time at which the eggs inoculated with MLD died were established.

(iii) RNA extraction and cDNA synthesis

Molecular characterization was conducted, which started with viral ribonucleic acid (RNA) extraction by using Kit (QIAGEN) following manufacturer instructions. Quantity and quality of isolated RNA was assessed by using Nano drop 2000 spectrophotometer (Thermo Scientific). Thereafter, complementary deoxyribonucleic acid (cDNA) was synthesized from the viral RNA using ProtoScript II First Strand cDNA Synthesis Kit (New England Biolab, NEB). The cDNA synthesis was achieved by reverse transcription of viral RNA using random primers supplier by the kit manufacturer (NEB). The 20 µl cDNA synthesis reaction was conducted in polymerase chain reaction (PCR) tubes (8 strips) in a thermal cycler (Biorad^{TM}) through a three-step reaction conditions: incubation at 25^{o}C for 5 minutes, reverse transcription at 42^{o}C for one hour, and enzyme inactivation at 80^{o}C for 5 minutes. Negative controls were treated with the same reaction conditions except that nuclease-free water was placed in a reaction volume instead of the viral RNA template. The cDNA samples were stored at -20^{o}C for further use.

(iv) Amplification of partial M and F genes of virulent NDV field isolate

Polymerase chain reaction was performed using two sets of primers (Table 5) to amplify partial region of M and F genes of virulent NDV isolate. The M primer was designed to target the matrix gene for general NDV detection (Wise *et al*., 2004); whereas, F primer was designed to identify virulent NDV strains by targeting the conserved region of 374 bp in length, which includes the F protein cleavage site in the NDV genome (Kim *et al*., 2007).

Table 5: Sequences of primers used for amplification of partial M and F genes of virulent NDV field isolate

Name	Primer	Sequences (5′–3′)	Amplicon size (bp[1])	An[2] (°C)	Reference
M	4100F/4220R	AGT GATGTGCTC GGA CCT TC CCT GAGGAGAGG CATTTG CTA	121	58	Wise *et al*. (2004)
F	4331F/5090R	GAGGTTACCTCYACYAAGCTRGAGA TCATTAACAAAYTGCTGCATCTTCCCWAC	750	58	Kim *et al*. (2007)

Legend: [1] Sequence length of nucleotides measured in base pairs (bp)
[2] The annealing temperature (An) in Celsius

The cDNA amplification was conducted by using Maxima SYBR Green/ROX qPCR Master Mix (2x) (Thermo Scientific) according to the manufacturer instructions. The 20 µl reaction volume in 96-well reaction plate (MicroAmp®Fast, Applied Biosystems) contained the primer final concentration 0.5 µM and the cDNA template final concentration ≤ 500 ng. The reaction conditions consisted of an initial holding denaturation at 94°C for 10 minutes followed by 40 cycles of denaturation at 94 °C for 15 seconds, primer annealing at 56°C for 30 seconds, an extension at 68°C for 1 minute and a final extension at 68°C for 5 minute. PCR reaction ran on a QuantStudio 6 Flex real-time (RT) PCR thermal cycler (Applied Biosystems). Polymerase chain reaction products were visualized under ultraviolet (UV) light after running for an hour on 1% weight/volume (w/v) agarose gel containing ethidium bromide in 1x tris-boric ethylenediaminetetraacetic acid (TBE) running buffer. The size of amplicons was compared with the 100 bp deoxyribonucleic acid (DNA) ladder (NEB).

(v) Bioinformatics and phylogenetic analysis of virulent NDV DNA sequences

The PCR product (~750 bp) of partial F protein gene of the isolate was sent to Inqaba Biotechnology (South Africa) for Sanger's dideoxy chain-termination sequencing method. Raw DNA sequences were electronically transmitted for analysis. The forward and reverse raw electropherogram sequences were manually edited and consensus sequence was generated in BioEdit v 5.0.6. Initially, the sequence was translated into protein, and an open reading frame was identified using ExPASy translate tool (Gasteiger *et al*., 2003) to determine the presence of multiple basic amino acids residues at 112-116 and phenylalanine residue at 117. Then, a multiple sequence alignment was performed with other reported reference genotypes by using the multiple sequence comparison by log-expectation (MUSCLE) algorithm in MEGA v 6 (Kanbach *et al*., 2004). Again, using maximum likelihood method in molecular evolutionary genetics analysis (MEGA) v 6 (Kanbach *et al*., 2004), a phylogenetic tree was constructed to establish the genetic relationship of the virulent

NDV isolate with the reference NDV that were previously reported in national center for biotechnology information (NCBI).

2.2.3 Experimental challenge of embryonated chicken eggs

The experiment involved Tanzania local chickens, and two exotic chickens: kuroiler (Fleming *et al*., 2016) and Sasso (Osei-Amponsah *et al*., 2012). A total of 355 chicken embryos (87 Sasso, 129 kuroiler and 139 local) were challenged in three experimental replicates. Furthermore, a total of 27 CEs were used as control where in each replicate, 3 CEs for each breed were challenged with PBS as mock infection. Parent chickens had the same history of vaccination against NDV. Chicken variability in susceptibility to virulent NDV was evaluated by inoculating 16-day-old chicken embryos (CEs) with 0.1 ml (10^3 MLD/ 0.1 ml) of virus suspension. The CEs were candled after 24 hours post challenge to detect early dead embryos, which may be a sign of bacterial contamination. Subsequent CEs candling was conducted after every 6 hours for further 96 hours post challenge to establish variability of CEs death time (DT) upon challenge with virulent NDV.

3.3 Association between genetic variants (GVs) of selected candidate genes and chicken embryos variation in susceptibility to virulent NDV

3.3.1 Genomic DNA extraction from chicken embryo tissues

Selective genotyping strategy was deployed as previously described (Darvasi & Soller, 1992; Sen *et al*., 2009). Therefore, chicken embryos from high (15%) and less (15%) susceptible cohorts were selected for genotyping. Genomic DNA was extracted from leg tissues using Quick-DNA Tissue/Insects Kit (Zymo Research) in accordance with manufacturer protocol. The quantity and quality of genomic DNA was measured by using nanodrop 2000 spectrophotometer (Thermo Scientific), and the integrity of genomic DNA was visualized under UV light following running on 1.5% (w/v) agarose gel containing ethidium bromide in 0.5% TBE running buffer. A total of 102 samples (40 kuroiler, 38 local chicken, and 24 sasso) were suitable for further analysis.

3.3.2 Genotyping of chicken Mx1 gene G2032A SNP

Genotyping of chicken Mx1 gene G2032A SNP was performed by PCR length polymorphism (PCR-LP) as previously described (Wang *et al*., 2012) with minor modification. The primers (+MX1SER: 5'-GCTCTCCTTGTAGGGAGCCAG-3'; +MXASN

5’-TAATAATAATAACCTCTCCTTGTAGGGAGCGAA-3’ and –MX1SERASN: 5’-GTGACTAATTCTGCTGGTCAGTAAC-3’) that were previously designed by Wang *et al*. (2012) were used for the PCR-LP. The primers amplify a DNA fragment that includes G2032A substitution in the coding region f chicken Mx1 gene.

The PCR conditions were initial denaturation at 94^{o}C for 2 minutes, which was followed by 40 cycles at 94°C for 30 seconds, 55°C for 1 minute, 72°C for 1 minute, and a final extension at 72°C for 7 minutes. After amplification, the PCR products were loaded on 2.5% (w/v) agarose gel and run in a 0.5% TBE buffer at 80 voltage (V) for 4 hours. Genotyping was possible because of the different size of PCR products generated by forward primers for alleles G (~199 bp) and A (~211 pb).

3.3.3 Genotyping of chicken Mx gene promoter

The polymerase chain reaction was conducted to amplify a DNA fragment of about 284 bp region of the 5’ untranslated region and partial promoter of the chicken Mx gene (Li *et al*., 2007). The selected primers (forward primer: 5’-ACCTGTGCCATCTGCCCTCTGA-3’ and reverse primer: 5’-CACAGCAAGGAGAAACAATTAACTACAT-3’) and PCR conditions were as previously described (Mishra *et al*., 2011). The amplification was conducted in reaction volume of 25 µl containing 0.2 µM of each primer and 12.5 µl of 2x Taq PCR Master Mix (NEB). The PCR reaction conditions were as follows: initial denaturation at 94°C for 5 minutes, followed by 40 cycles of denaturation at 94°C for 1 minute, annealing at 58.5°C for 30 seconds and extension at 72°C for 1 minute, and final extension at 72°C for 5 minutes. The PCR reaction ran on a QuantStudio 6 Flex real-time (RT) PCR thermal cycler (Applied Biosystems). Quality of PCR products was evaluated by running on 1% (w/v) agarose gel containing ethidium bromide in 0.5 X TBE buffer at 100 V for an hour.

Polymerase chain reaction products were sent to Inqaba Biotech for Sanger sequencing. Raw sequences trace files were electronically transmitted for analysis. Raw sequences were trimmed using CLC Genomics workbench v.3.0.8 and consensus sequences were generated. A total of 88 sequences (24 kuroiler, 32 local chicken and 32 Sasso) were further analysed. Multiple sequence alignment was done using MUSCLE algorithm in MEGA v.6 (Tamura *et al*., 2013) to identify polymorphic sites. Variant calling was done using CLC Genomics workbench v.3.0.8.

3.3.4 Genotyping of LEI0258 microsatellite marker

The PCR amplification of LEI0258 marker was done using a forward primer (CAJF01F) 5'-TCGGGAAAAGATCTGAGTCATTG-3' and reverse primer (CAJF01R) 5'-TGATTTTCAGATCGCGTTCCTC-3' (Fulton *et al*., 2006). The primers bind just outside of the LEI0258 binding region including entire region encompassed by the LEI0258 primers. The LEI0258 primers are; LEI0258-F: CACGCAGCAGAACTTGGTAA forward and LEI0258-R: AGCTGTGCTCAGTCCTCAGTGC reverse (McConnell *et al*., 1999). The PCR reaction volume was 25 µl, which contained 0.1 µM of forward and reverse primers and 12.5µL of 2x Taq PCR MasterMix (New England Biolabs, NEB), and the PCR reaction conditions were: initial denaturation at 95^{o}C for five minutes, which was followed by 35 cycles of denaturation at 95°C for 30 seconds, annealing at 61°C for 30 seconds and extension at 72°C for 30 seconds, and final extension at 72°C for 5 minutes. The PCR products were confirmed by running on a 2.5% agarose gel containing ethidium bromide for 2 hours. The gel was exposed to UV light to visualize the amplicons, and a 100 bp DNA ladder (New England Biolabs, NEB) was used for comparison with amplified fragments size. PCR products were purified by using QIAquick PCR Purification Kit (QIAGEN) before shipment to Inqaba Biotechnology (South Africa) for Sanger sequencing. Homozygous samples were selected, and heterozygous samples with unique alleles were used after separation of alleles. Each of the DNA samples was sequenced in both forward and reverse direction.

Initially, raw sequences were trimmed, and consensus sequences were generated with the use of CLC Genomics workbench v.3.0.8 (QIAGEN). Then, sequences upstream and downstream of LEI0258 primers (McConnell *et al*., 1999) were trimmed. After pre-processing, a total of 75 (29 kuroiler, 29 Local chicken and 17 Sasso) DNA sequences were suitable for downstream analysis. Sequences were aligned with MUSCLE algorithm in MEGA v6 (Tamura *et al*., 2013) to detect polymorphic sites (i.e., SNPs and Indels) in upstream and downstream flanking sequences of tandem repeats. The neighbor-joining method in MEGA v6 was used to construct phylogenetic trees to visualize clustering of the DNA sequences. Two repeat elements, a 13 bp repeat of "CTATGTCTTCTTT" and a 12 bp repeat of "CTTTCCTTCTTT" were counted with the use of functions in SeqKit v0.10.1 (Shen *et al*., 2016). Polymorphisms at repeats (R13/R12) and flanking regions (SNPs and Indels) were summarized in a table.

3.4 Data analyses

One-way analysis of variance (ANOVA) was conducted to assess difference in BWG and average antibody titers between chicken types. Also, the Student t-test was used to test difference between BWG between control and challenge birds, and to test difference between average antibody titers between two time points (days 10 and 21 post challenge). The relationship between BWG and antibody titers was evaluated by performing correlation test.

Moreover, the within breed chicken embryos coefficient of variation (Cv) in susceptibility to virulent NDV challenge was tested by using Krishnamoorthy and Lee's (Krishnamoorthy & Lee, 2014) modified signed-likelihood ratio test. Pearson's chi-squared test of independence (association) and likelihood ratio (LR) was conducted, specifically, to test for a null hypothesis that chicken embryos variability in susceptibility to virulent NDV infection is independent of chicken Mx1 gene genotypes (AA, AG and GG) or alleles (A and G). On the other hand, population genetics parameters like Hardy-Weinberg equilibrium, pairwise linkage disequilibrium, and its correlated association analysis were performed by using SNPStats a web tool for SNP analysis of chicken Mx gene promoter (Solé *et al*., 2006).

It is well established that allele frequency at a particular locus in a random mating population is expected to be increased by natural selection if it plays a crucial role for survival of individuals in the environment (Lwelamira *et al*., 2008), and therefore LEIO258 marker alleles with frequency ≥ 3 were considered for association analysis. The association of MHC haplotypes as determined by LEI0258 marker alleles with chicken embryos susceptibility to virulent NDV challenge disease susceptibility was conducted by inference technique as previously described (Labouriau *et al*., 2008; Schou *et al*., 2010). Briefly, groups of marker alleles (MAGs) were established to represent most possible MHC haplotypes because more than one marker allele might be in linkage disequilibrium (LD) with a particular MHC haplotype (Labouriau *et al*., 2008).

All descriptive and inferential statistical analyses like Pearson's chi-squared test of independence of genotype and allele frequencies and likelihood ratio tests were conducted by use of R software (version 3.3.3, The R Foundation for Statistical Computing). The test was considered statistical significant give that $P < 0.05$.

3.5 Ethical statement

The experiment was conducted in compliance with the guidelines on the humane treatment of Laboratory animals as stipulated in the Tanzania animal welfare act, 2008.

CHAPTER FOUR

RESULTS AND DISCUSSION

4.1 Results

4.1.1 Average body weight and body weight gain post ND vaccination

Weekly means ± standard errors of body weight and BWG in grams (g) following vaccination are summarized in Table 6, and body weight raw data is in Appendix 1.

Table 6: Weekly average body weight and body weight gain (BWG) in gram (g) post ND vaccination of kuroilers, broilers and local Tanzanian chicken

Chicken type	Means body weight ± standard Errors							
	W1 (g)	**W2 (g)**	**W3 (g)**	**W4 (g)**	**W5 (g)**	**W6 (g)**	**W7 (g)**	**BWG (g)[1]**
Broiler	363.93± 13.60	670.79± 21.46	1114.31± 27.21	1407.39± 25.12[a]	1830.45± 35.79	2198.54± 41.39	2528.06± 48.95[a]	1120.67± 29.99[a]
Kuroiler	142.70± 7.21	237.69± 18.76	371.60± 38.12	476.06± 14.27[b]	609.10± 17.29	743.82± 20.48	886.80± 25.63[b]	410.74± 13.34[b]
Local	146.95± 7.35	253.18± 34.72	348.42± 13.72	471.57± 16.63[b]	614.65± 20.68	747.84± 24.82	880.07± 29.67[b]	408.50± 15.34[b]

Legend: Means of the same column bearing different superscript are significantly different from each other (Tukey's HSD, $P<0.05$); W1, W2, W3, W4, W5, W6, and W7=, body weight at 1, 2, 3, 4, 5, 6 and 7 weeks of age; g is grams.

The broilers had the highest growth performance throughout the experiment followed by kuroilers. The kuroiler chickens had higher ($P > 0.05$) body weight than local chickens except at the first three weeks (W1, W2, and W3), where the body weight of local chickens were higher ($P > 0.05$) than that of kuroiler chickens. The BWG was higher ($P > 0.05$) in the control group than in the challenge group (Fig. 3).

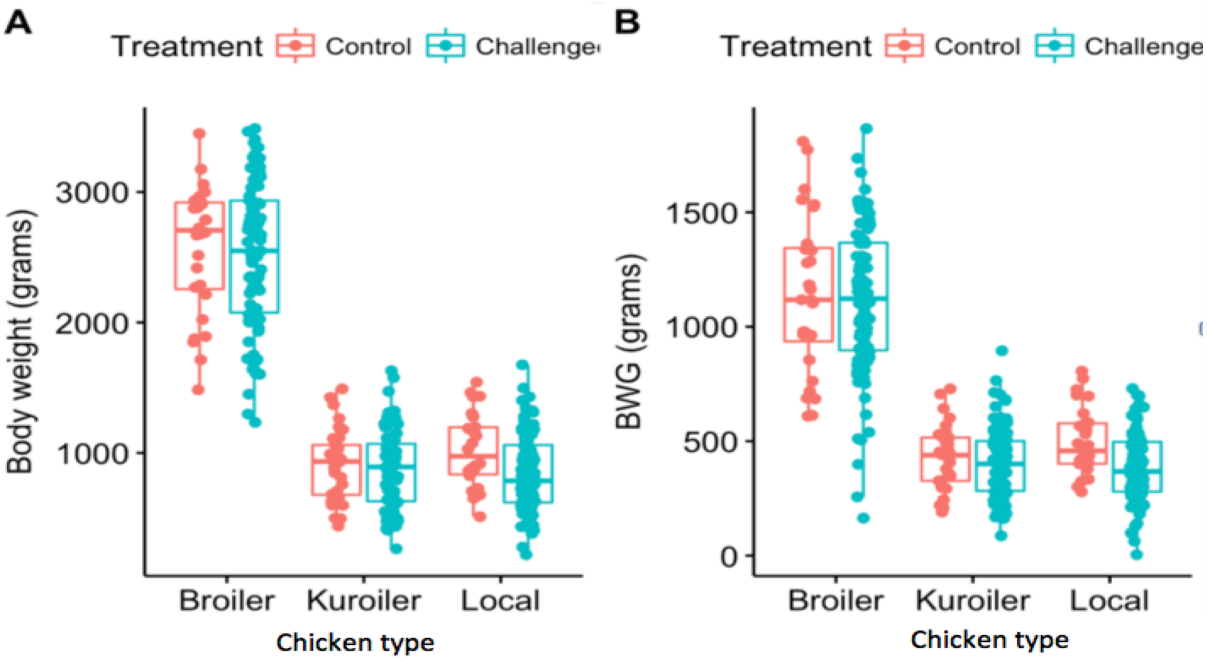

Figure 3: Chicken growth performance (A) and body weight gain, BWG, (B) of kuroilers, broilers and local Tanzanian chicken

4.1.2 Antibody titres post ND vaccination in kuroiler, broiler and local Tanzanian chicken

Means ± standard errors of antibody response against NDV at two time points (days 10 and 21 post vaccination) are presented in Table 7, and antibody titre raw data is found in Appendix 2. The antibody titers were higher in kuroiler and local chickens compared to antibody titers in broiler chickens. The antibody titers of kuroiler and local chickens at 10 days post challenge were different ($P < 0.05$) from that of broilers. Kuroiler chickens had higher ($P > 0.05$) antibody titer than local chickens at two time points. As expected, antibody titers were different ($P < 0.0001$) between days 10 and 21 post challenge. Mean (3.91 ± 0.04) antibody titer at 21-days post challenge was higher than the mean (3.69 ± 0.04) antibody titer at 10-days post challenge (Fig. 4). Furthermore, there was positive correlation of antibody titers between days 10 and 21 post challenge. The correlation of antibody titer between day 10 and 21 was intermediate in broiler ($r = 0.52$; $P = 4.1e\text{-}5$) and local ($r = 0.4$; $p=0.006$) chickens as compared to weak correlation in kuroiler ($r = 0.1$; $P = 0.4$) chickens.

Table 7: Antibody titres at days 10 and 21 post ND vaccination in kuroilers, broilers, and local Tanzanian chicken

	Time (days) post challenge	Treatment	Breed		
			Broiler	Kuroiler	Local
Means antibody titers ± standard error	10	Challenged	3.53±0.06[a]	3.81±0.06[b]	3.73±0.07[b]
		Control	2.11±0.09[a]	2.35±0.11[a]	2.38±0.09[a]
	21	Challenged	3.80±0.06[a]	4.00±0.08[b]	3.90±0.04[ab]
		Control	2.40±0.21[a]	2.11±0.17[a]	2.10±0.11[a]

Legend: [1]Total number of chickens that were vaccinated for each breed; Means of the same row bearing different superscript were significantly different from each other (Tukey's HSD, $P < 0.05$)

The antibody titers were different ($P < 0.0\ 001$) between control and challenged group in all chicken types at the two time points (Fig. 4). The antibody titers were not different ($P = 0.37$) in the three chicken types at days 10 and 21 in the control group (Fig. 4).

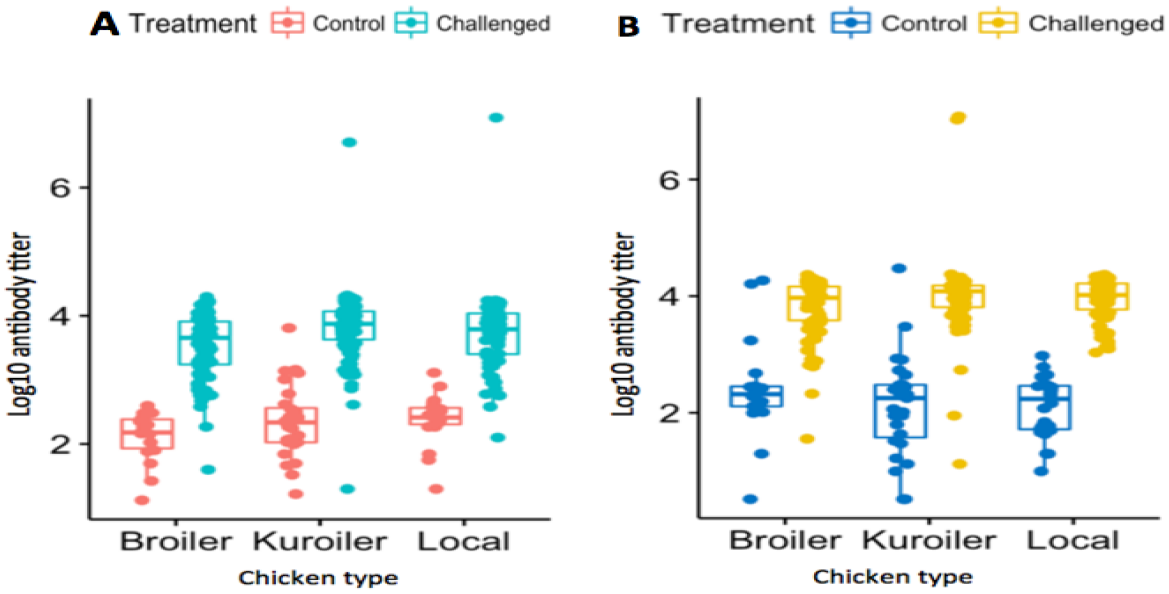

Figure 4: Antibody titres at days 10 (A) and 21(B) post ND vaccination in kuroilers, broilers and local Tanzanian chicken

4.1.3 Biological and molecular characterization of the virus

Hemagglutination test confirmed the presence of the hemagglutinating agent in the homogenate. The isolate reacted with monospecific antiserum specific against NDV confirming the presence of NDV. The MDT of 10-day-old embryonated chicken eggs infected with the isolate was 55.5 hours with the death time ranges from 36 to 72 hours. The presence of NDV in the isolate was further confirmed by amplification of matrix gene (~121 bp, Fig. 5). The molecular analysis of F protein gene cleavage site revealed the presence of the motif "R-R-Q-K-R-F" suggestive of virulent strain.

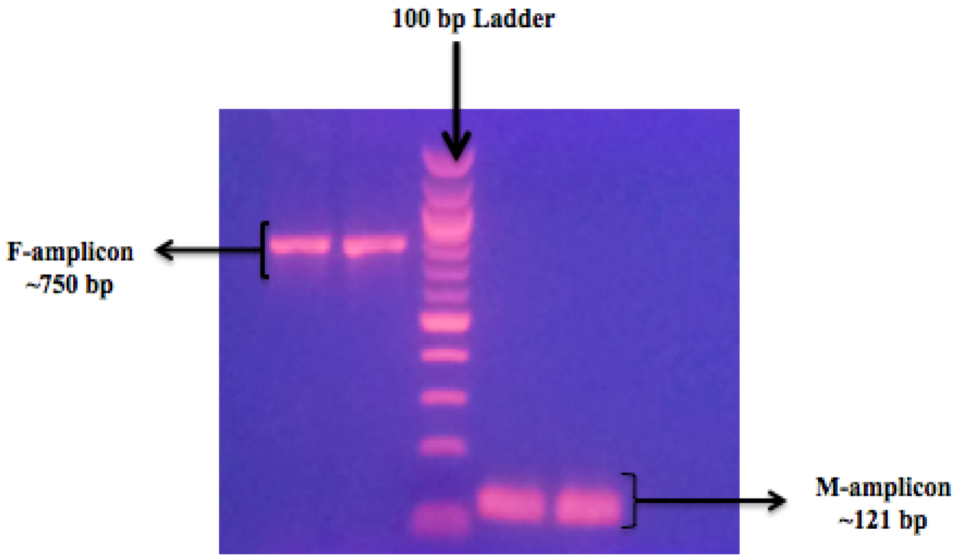

Figure 5: Gel image of polymerase chain reaction (PCR) product of partial F and M gene of virulent NDV field isolate from live bird market in Morogoro, Tanzania

Genetic relatedness and phylogenetic analysis of the partial F-gene sequences (~750 bp, Fig. 4) of the vNDV isolate showed that the isolate clustered together with other Tanzanian isolate of year 2010 and Indian isolate of year 2015 (Fig. 6).

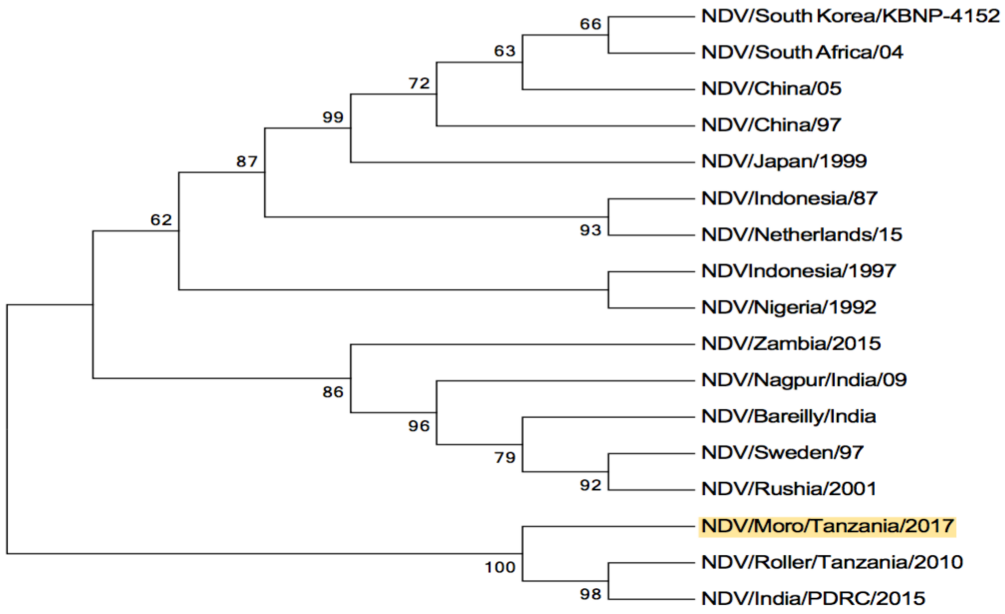

Figure 6: The genetic relatedness of vNDV (highlighted in yellow) isolated from live bird market in Morogoro region of Tanzania in 2017 with other reference genotypes. Unrooted ML phylogram was constructed targeting partial fusion protein gene nucleotides (~700 bp) of NDV. The bootstrap confidence limit values >60% after 1,000 simulation samplings are indicated on the tree nodes.

4.1.4 Chicken embryos survival variability following challenge with virulent NDV

Infecting 16-day-old embryonated eggs assessed chicken embryos variability in susceptibility to virulent NDV. Death time post infection was used as a measure of variations in susceptibility to the infection. The death time statistics of chicken embryo upon infection with virulent NDV is summarized in Table 8. Also, as expected, the death time within a breed was highly variable, and the coefficient of variation between breeds was different ($P < 0.05$).

Table 8: Statistics of local Tanzanian, kuroiler and Sasso chicken embryos survival time upon challenge with minimum lethal dose of virulent Newcastle disease virus

Chicken type	Mean (hrs.)	SEM[1]	Median[2] (hrs.)	25th percentiles (hrs.)	75th percentile (hrs.)	IQR[3] (hrs.)
Kuroiler	62.3	1.5	66	54	78	24
Local	60.8	1.1	66	54	66	12
Sasso	76.5	2.1	78	66	90	24

Legend: [1] Standard error of the mean
[2] The median, which represent 50th percentiles
[3]Interquartile range

4.1.5 Polymorphism of chicken Mx gene G2032A SNP and survival variability

A PCR-LP using two forward primers (+MX1SER and +MX1ASN) and a reverse primer (-MX1SERASN) produced two different size of PCR products of A and G allele (Fig. 7).

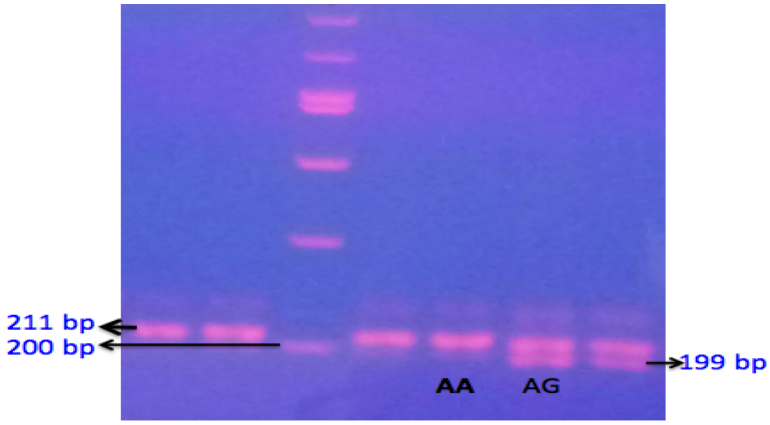

Figure 7: Polymerase chain reaction length polymorphism (PCR- LP) genotyping of chicken Mx1 gene G2032A SNP. Example of PCR-LP products separation indicating alleles A (211pb) and G (199 pb). GG: homozygous G allele; AA: homozygous A allele; AG: heterozygous

An allele A is considered resistant, whereas an allele G is considered susceptible, and likewise, chickens with genotype AA are considered resistant, whereas birds with genotype GG are considered susceptible (Ko *et al*., 2002b, 2004).

The frequency of an allele A was higher compared to frequency of an allele G in all chicken breeds (Table 9). The frequency of an allele A was highest in Sasso (0.66) as compared to kuroiler and local chicken, which both had allele A frequency of 0.64. Furthermore, the allele frequency was associated ($P < 0.05$) with the chicken embryos variation in susceptibility to virulent NDV infection except for Sasso chicken embryos (Table 9). The alleles and genotypes raw data for chicken Mx gene G2032A SNP is found in Appendix 3.

Table 9: Allele frequencies of the Mx gene G2032A SNP in local Tanzanian, kuroiler and Sasso chicken breeds and associations with chicken embryos susceptibility to virulent NDV infection

Breed	Allele	Susceptibility		χ^2, P value	LR, P value
		H	L		
Kuroiler	A	20 (0.50)	31 (0.78)	$X^2 = 6.545$	LR = 6,67
	G	20 (0.50)	9 (0.22)	P = 0.011	P = 0.009
Local	A	19 (0.50)	30 (0.79)	$X^2 = 6.951$	LR = 7.105
	G	19 (0.5)	8 (0.21)	P = 0.008	P = 0.008
Sasso	A	17 (0.71)	15 (0.63)	$X^2 = 0.375$	LR = 0.376
	G	7 (0.29)	9 (0.37)	P = 0.540	P = 0.539

Legend: χ^2 is Pearson's chi-squared test of independence (association), LR is likelihood ratio, H is highly susceptible, L is less susceptible and P values <0.05 considered statistically significant

Also, at the genotype level, the homozygous AA genotype had higher frequency compared to homozygous GG genotype (Table 10). Pearson's chi-squared test of independence and likelihood ratio tests demonstrated an association ($P < 0.05$) between chicken Mx1 gene G2032A genotypes and chicken embryos variation in susceptibility to virulent NDV infection. The frequency of homozygous AA genotype was higher in less susceptible chickens, whereas frequency of homozygous GG genotype and heterozygous AG genotype were higher in high susceptible chickens. However, similar trend was not observed in Sasso chicken embryos (Table 10).

Table 10: Genotype frequencies of the Mx gene G2032A SNP in local Tanzanian, kuroiler and Sasso chicken breeds and associations with chicken embryos susceptibility to virulent NDV infection

Breed	Genotype	Susceptibility		χ^2, P value	LR, P value
		H	L		
Kuroiler	AA	6 (0.30)	11(0.55)	$X^2 = 7.529$, P = 0.023	LR = 8.869, P = 0.007
	AG	8 (0.40)	9 (0.45)		
	GG	6 (0.30)	-		
Local	AA	4 (0.21)	13 (0.68)	$X^2 = 8.69$, P = 0.013	LR = 9.093, P = 0.011
	AG	11 (0.58)	4 (0.21)		
	GG	4 (0.21)	2 (0.11)		
Sasso	AA	6 (0.50)	4 (0.34)	$X^2 = 0.733$, P = 0.693	LR = 0.737, P = 0.692
	AG	5 (0.42)	7 (0.58)		
	GG	1 (0.08)	1 (0.08)		

Legend: χ^2 is Pearson's chi-squared test of independence (association), LR is likelihood ratio, H is highly susceptible, L is less susceptible and P values < 0.05 considered statistically significant

4.1.6 Polymorphism of chicken Mx gene promoter and survival variability

A total of five single nucleotide polymorphic sites (SNPs) were observed in the present study (Table 11). All SNPs were previously reported when the same (284 bp) promoter region of chicken Mx gene was sequenced (Mishra *et al*., 2011). Generally, the observed and expected heterozygosity for all SNPs were at the same levels (Table 11). Furthermore, all the SNPs had no interaction between the response variable (susceptibility) and covariate (breed), with exception of SNP3. Additionally, SNP3 was not associated with chicken embryos variability in susceptibility to virulent NDV infection, and therefore SNP3 was removed from further analysis to allow breeds merging. The alleles and genotypes raw data for chicken Mx gene promoter variants is found in Appendix 4.

Table 11: Single nucleotide polymorphic sites observed in local Tanzania, kuroiler, and Sasso chicken

Markers	Position	H_O	H_E	Allele change
SNP1	91st	0.34	0.28	A > T
SNP2	107th	0.34	0.32	C > G
SNP3	113th	0.34	0.38	C > T
SNP4	194th	0.28	0.30	G > A
SNP5	231st	0.31	0.37	T > C

Legend: H_O is observed heterozygosity; H_E is expected heterozygosity. Note: Position is polymorphic site correspond to 1 to 284 positions along the amplified product length from 5' to 3'

(i) The Hardy-Weinberg equilibrium analysis

The SNPs were tested for agreement with Hardy-Weinberg equilibrium (HWE). All SNPs were in consistence with the Hardy-Weinberg principle, HWE (P) > 0.05 (Table 12). Also, the minor allele frequencies in high (H) and less (L) susceptible cohorts were greater than 0.05 (Table 12).

Table 12: Allele frequency of polymorphic sites of promoter region of chicken Mx gene and association with chicken embryos susceptibility to virulent NDV infection

Markers	Allele	Susceptibility		χ^2, P value	LR, P value	HWE (P)	MAF
		H	L				
SNP1	A	59 (0.78)	85 (0.85)	0.02	0.01	1	0.16
	T	17 (0.22)	15 (0.15)	0.89	0.09		
SNP2	C	58 (0.76)	84 (0.84)	1.64	1.62	1	0.20
	G	18 (0.24)	16 (0.16)	0.20	0.2		
SNP4	G	58 (0.76)	85 (0.85)	2.14	2.12	0.49	0.19
	A	18 (0.24)	15 (0.15)	0.14	0.14		
SNP5	T	58 (0.76)	75 (0.75)	0.04	0.04	0.14	0.24
	C	18 (0.24)	25 (0.25)	0.84	0.84		

Legend: χ^2, Pearson's chi-squared test of independence; , LR, likelihood ratio; HWE (*P*), *P* value of the Hardy-Weinberg equilibrium test; H, high susceptible chicken embryo group; L, less susceptible chicken embryo group; and MAF, minor allele frequency

(ii) SNPs allele and genotype frequency

The allele and genotype frequencies of 4 SNPs (SNP1, SNP2, SNP4 and SNP5) are presented in Tables 12 and 13. At the allelic level, the results indicated that not association between SNPs and chicken embryos susceptibility to virulent NDV challenge. However, at genotypic level, SNP4 (LR = 6.97, P < 0.05) was significantly associated with chicken embryos susceptibility to virulent NDV infection (Table 13).

Table 13: Genotype frequency of polymorphic sites of chicken Mx gene promoter and association with chicken embryos susceptibility to virulent NDV infection

Markers	Genotype	Susceptibility		χ^2, P value	LR, P value
		H	L		
SNP1	AA	24 (0.63)	35 (0.7)	4.11 0.13	5.20 0.07
	AT	11 (0.29)	15 (0.3)		
	TT	3 (0.08)	-		
SNP2	CC	23 (0.61)	34 (0.68)	4.13 0.13	5.23 0.07
	CG	12 (0.32)	16 (0.32)		
	GG	3 (0.08)	-		
SNP4	AA	4 (0.11)	-	5.52 0.04	6.97 0.03
	AG	10 (0.26)	15 (0.3)		
	GG	24 (0.63)	35 (0.7)		
SNP5	CC	4 (0.11)	4 (0.08)	0.67 0.72	0.67 0.72
	CT	10 (0.26)	17 (0.34)		
	TT	24 (0.63)	29 (0.58)		

Legend: χ^2, Pearson's chi-squared test of independence; LR, likelihood ratio

(iii) Linkage disequilibrium and haplotypes frequency

Results of linkage disequilibrium (LD) of 5 SNPs are shown in Table 14. All the SNPs were in LD (P < 0.05) with the exception of SNP3 (P > 0.05), which was in equilibrium with other SNPs (Table 14).

Table 14: The linkage disequilibrium r statistic for five SNPs reported in the present study

	SNP1	SNP2	SNP3	SNP4	SNP5
SNP1	-	0.96	-0.28	0.98	0.83
SNP2	-	-	-0.29	0.94	0.79
SNP3	-	-	-	-0.28	0.07
SNP4	-	-	-	-	0.81
SNP5	-	-	-	-	-

Haplotype analysis of four SNPs that were in LD generated 4 haplotypes (Table 15). Haplotype group "ACGT" had highest haplotype frequency (0.74), and the lowest haplotype frequency (0.01) was observed in haplotype group "AGGT". Haplotype group "ACGC", which had a frequency of 0.06 was associated ($P<0.05$) with chicken embryos susceptibility to virulent NDV infection (Table 15).

Table 15: Haplotype analysis of four polymorphic sites of chicken Mx gene promoter that are in LD and association with chicken embryos susceptibility to virulent DND infection

	Haplotypes				Frequencies			OR (95% CI)	P value
No	**SNP1**	**SNP2**	**SNP4**	**SNP5**	**Total**	**H**	**L**		
1	A	C	G	T	0.74	0.74	0.74	1.00	-
2	T	G	A	C	0.06	0.22	0.15	0.54 (0.21 – 1.36)	0.2
3	A	C	G	C	0.06	0.01	0.10	9.18 (1.06 – 79.43)	0.042
4	A	G	G	T	0.01	0.01	0.01	0.73 (0.04 – 12.88)	0.83

Legend: CI, confidence interval; OR, odds ratio; H, high susceptible chicken embryo group; L, less susceptible chicken embryo group; and No, serial number

4.1.7 Polymorphisms of LEI0258 microsatellite marker and relationship with survival variability

As described by Fulton *et al*., (2006); two levels of polymorphisms were detected: two repeats: R13 (ATGTCTTCTTTCT) and R12 (TTCCTTCTTTCT), and SNPs and indels in the upstream and downstream flanking sequences (Appendix 5). A total of 9 SNPs and indels were detected. Three SNPs and two indels were detected in the downstream flanking sequences. The SNPs in downstream flanking sequence were found at positions 3, 37 and 44 bp, and largest deletion (ATTTTGAG) at positions 21-28 bp in the downstream flanking sequences was also detected. Moreover, three SNPs at position 2, 12, and 30 bp and an indel (TT) at positions 31-32 bp in the upstream flanking sequences were detected. More

importantly, some SNPs were common based on phenotype (susceptibility). For example, the C > T SNP at position to in the upstream flanking region was observed in low susceptibility DNA sequences. Furthermore, there was a correlate between pattern of Indels and/or SNPs and allele size (Appendix 5).

On the other hand, number of repeats (R13 and R12) was highly variable. R13 appeared 1 to 5 times, whereas R12 appeared 3 to 13 times. The mean number of alleles observed in the present study was seven, with the higher number of alleles observed in kuroilers (8) and local Tanzanian chicken (8) and lower number of alleles observed in Sasso (5). The allelic sizes ranged from 194 to 452 pb (Appendix 5).

The LEI0258 DNA sequences were aligned using MUSCLE algorithm in MEGA v6 and the neighbor-joining method was used to establish percentage divergence for multiple sequence alignment. Sequences for each breed (kuroiler, Sasso, and local chickens) were analyzed separately. The multiple alignments that were saved in mega format were used to construct unrooted trees depicting the relationship between haplotypes and susceptibility in chicken embryos challenged with virulent NDV. The phylogenetic trees are presented in Fig. 8, Fig. 9 and Fig. 10.

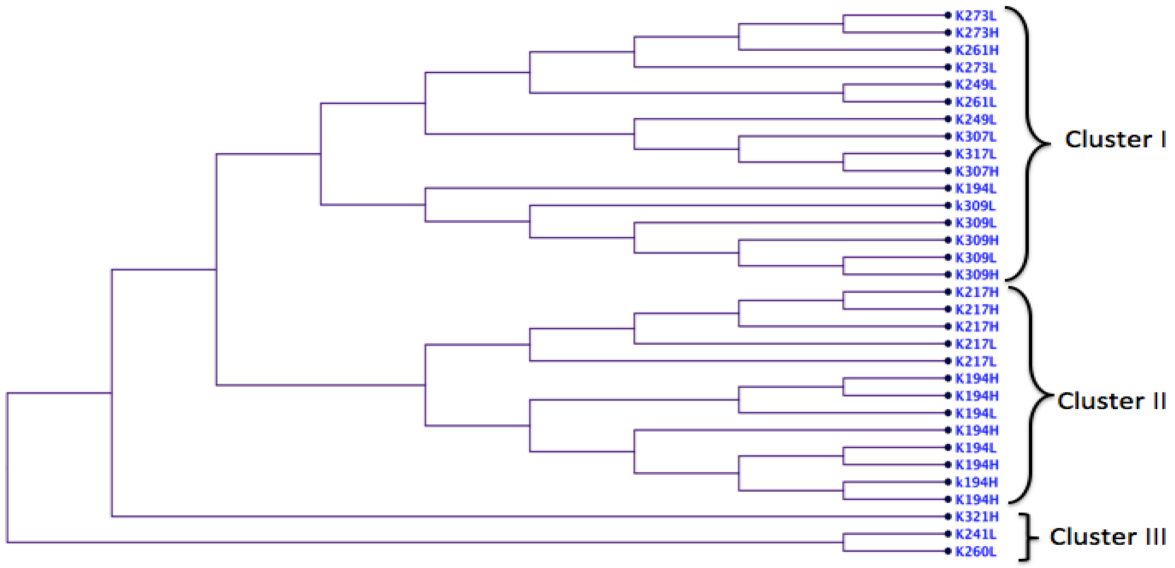

Figure 8: Neighbor-joining phylogenetic tree for kuroiler LEI0258 marker DNA sequences generated by using full likelihood distance and general reversible model. In branch name: K is kuroiler; the number is the allelic size in base pair (bp); H is high susceptible, and L is less susceptible

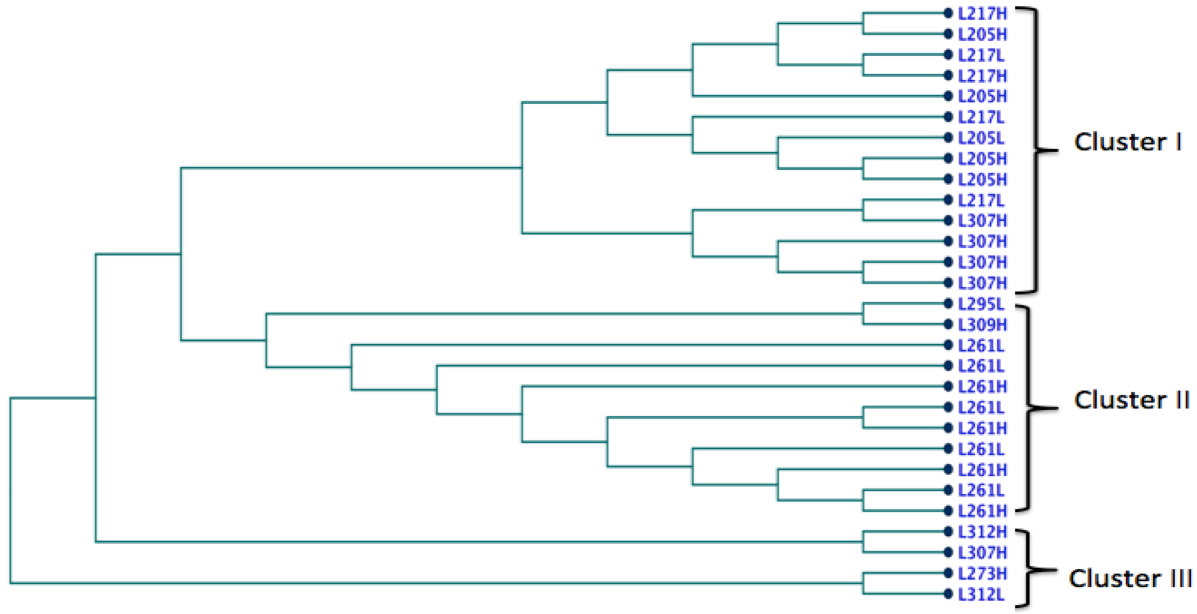

Figure 9: Neighbor-joining phylogenetic tree for local chicken LEI0258 marker DNA sequences generated by using full likelihood distance and general reversible model. In branch name, the first L is local chicken; the number is the allelic size in base pair (bp); H is high susceptible, and L is less susceptible

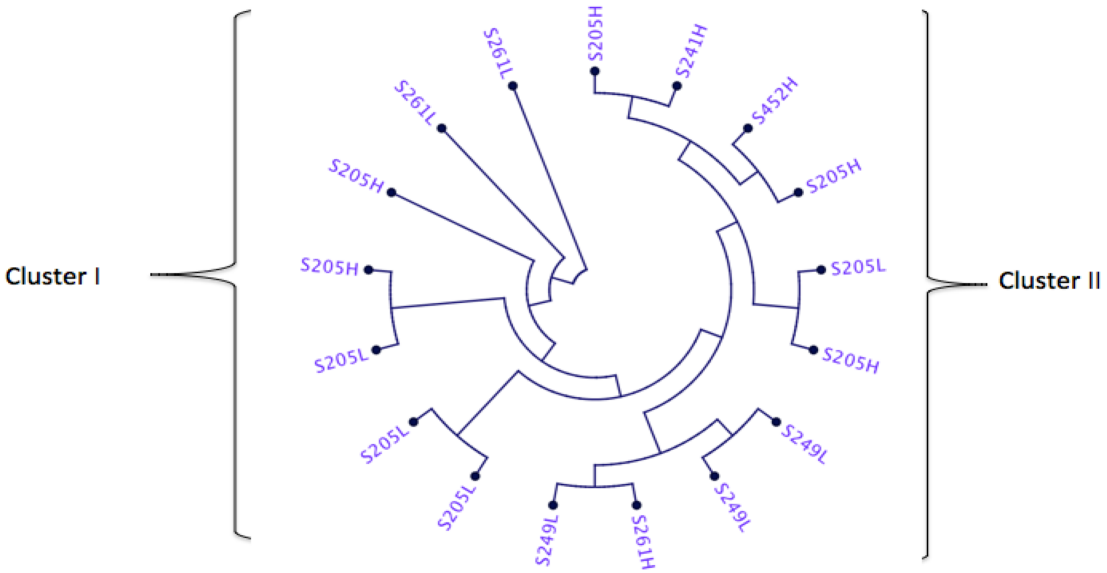

Figure 10: Neighbor-joining phylogenetic tree for Sasso LEI0258 DNA sequences generated by using full likelihood distance and general reversible model. In branch name, S is Sasso; the number is the allelic size in base pair (bp); H is high susceptible, and L is less susceptible

From phylogenetic analysis it is evident that the clustering of LEIO258 marker alleles was based on the levels of chicken embryos variation in susceptibility to virulent NDV challenge (Fig. 8, Fig. 9 and Fig. 10). For the kuroiler chicken (Fig. 8), 69% of LEI0258 marker alleles in cluster I constituted of less susceptible; whereas, the same percentage constituted of high susceptible in cluster II. In Tanzania local chicken (Fig. 9), 71% of cluster I LEI0258 marker alleles constituted of high susceptible as compared to 55% of cluster II of the same breed that constituted of less susceptible. The same trend was observed for Sasso (Fig. 10), where LEI0258 marker alleles clustered based on the levels of susceptibility with 71% of LEI0258 marker alleles in cluster I comprised of less susceptible as compared to 60% of LEI0258 marker alleles in cluster II that comprised of high susceptible.

However, upon testing of MAGs with chicken embryos variation in susceptibility to virulent NDV challenge, the association was not established with any of the selected MAG ($P > 0.05$).

4.2 Discussion

4.2.1 Growth performance and antibody responses following ND vaccination

Kuroiler, broiler and local Tanzanian chickens were raised for seven weeks. Chicks were raised for four weeks before vaccination to allow waning of maternal antibodies that would affect the effectiveness of the vaccine (Gharaibeh & Mahmoud, 2013).

The broilers average body weights of 1407.39 ± 25.12 g and 2528.06 ± 48.95 g at four and seven weeks of age, respectively reported in the present study are higher as compared to the previous reports. In the previous report in Tanzania, the mean broilers body weights were 396 g and 1255 g at four and eight weeks of age, respectively (Munisi *et al*., 2015). In another study that was conducted in Nigeria, the broiler average body weight was 2360 g at 20 weeks of age (Adeleke *et al*., 2011). The higher average broilers body weight observed in the present study may be explained by breed difference of the broilers involved in the studies. Also, the differences may be explained by feed content variations between the studies. For example, four weeks old broilers that were feed with feed supplemented with baobab seed oil cake had body weight of 1266 g, which is comparable to the broilers mean body weight of 1407.39 ± 25.12 g reported in the present study (Chisoro *et al*., 2018).

Kuroiler is dual-purpose chicken raised for egg and meat production (Dessie & Getachew, 2016). Like local chickens, kuroilers can thrive under harsh tropical environmental conditions, and they can scavenge for nutrition needs just like local chickens (Isenberg, 2008; Dessie & Getachew, 2016). The breed outperforms local chickens in terms of meat and egg production (Isenberg, 2008; Yakubu & Ari, 2018). However, in the present study kuroilers (886.80 ±25.63 g) and local Tanzania chicken (880.07 ±29.67 g) body weights were comparable ($P > 0.05$). The discrepancy may be explained by differences in chicken breeds involved in the studies. Local Tanzania chickens are not well characterized and due to lack of genetic resources conservation programs, maybe local chicken involved in the present study has acquired high growth performance genetic materials from improved breed through interbreeding. Also, feed regime used in the present study maybe had significant contribution on growth performance observed in kuroilers and local Tanzanian chickens. This is corroborated by higher body weights observed in the present study as compared to the previous reports. For example, Egyptian chicken (Mandarah) mean body weights at four and seven were 299 g and 747 g, respectively (Taha *et al*., 2012). In another study conducted in

Nigeria to evaluate growth characteristic of kuroiler and Nigerian local chicken (Fulani), at six weeks of age the mean body weight of kuroiler and Fulani was 450 g and 228 g, respectively (Yakubu & Ari, 2018). In another study conducted in Tanzania involving local chickens, the mean body weights at four and seven weeks of age were 151 g and 419 g, respectively.

Although not supported by empirical studies, kuroilers are said to be resistant to infectious diseases (Sharma *et al*., 2015; Fleming *et al*., 2016). However, in the present study kuroiler and local chicken antibody titres were comparable. Furthermore, the observed antibody titers in the present study are relatively higher compared to the previous findings in other parts of the world. For example, Luo *et al*. (2013), reported mean antibody titer of 3.3 at 41 days after second immunization among Chines-yellow broiler chickens. Another report found the mean antibody titer of 3.2 at 15 days post challenge among commercial meat chicken type (Tabidi *et al*., 2004).

Generally, although results showed differences between vaccinated and control groups, results could not give clear cut differences on variation in susceptibility, probably because a less virulent strain of NDV was used and the housing environment might have created some confounding factors as previously described (Schilling *et al*., 2018). Therefore, in subsequent experiment challenging chicken embryos with virulent NDV was thought because it would require a very large sample size to establish clear-cut difference in susceptibility by vaccinating chicken with lentogenic strain of NDV.

4.2.2 Assessment of chicken embryos survival variability upon challenge with virulent NDV in kuroiler, Sasso and local Tanzanian chicken

The second phase of the present research was to study genetic mechanisms for chicken variations in susceptibility to virulent NDV challenge by using chicken embryo model. Initially, the local NDV field isolate was characterized to confirm virulence of the strain. Therefore, ten-day–old embryonated chicken eggs were infected with the isolate, and MDT was 55.5 hours, which was within MDT range of < 60 hours when 9 to 11-day old embryonated chicken eggs are infected with virulent NDV (Alexander, 2000). Furthermore, using molecular approach, the NDV isolate was first confirmed by amplification of ~121 bp of the NDV matrix gene using gene-specific pair of primers (M-primers). Then, the NDV field isolate was definitively confirmed to be virulent strain by detection of multiple basic

amino acid motif ("112-R-R-Q-K-R-F-117") at F protein cleavage site following the sequencing of PCR product (~750 bp) that was generated by amplifying the intergenic region between M and F genes of the NDV. In addition, the isolate was highly genetically related to other isolates that were previously reported in other parts of the world (Fig. 6). The characterized virulent NDV field isolate provided a platform to test chicken embryos variability in susceptibility to the virus in 3 chicken breeds: Tanzania local chickens, Kuroilers, and Sasso.

Chicken embryos variability in susceptibility to virulent NDV challenge assessed by infecting 16-day-old embryonated eggs. Death time post challenge was used as a measure of variations in susceptibility. As expected, the death time within a breed was highly variable. The high variability in death time within a breed may be explained by heterogeneity nature of the study populations. The three breeds under study (kuroiler, Tanzania local chicken and Sasso) were outbred populations, and therefore individual genetic differences within a breed are common.

4.2.3 Association between genomic polymorphisms of selected candidate genes and chicken embryos variation in susceptibility to virulent NDV

In the last phase of the research, a combination of candidate gene and selective genotyping approaches were deployed to investigate polymorphisms of selected candidate genes/marker (Mx1 and LEI0258) and association with chicken embryos susceptibility to virulent NDV challenge. Chicken embryos were initially challenged with MLD of virulent NDV suspension and information was continuously gathered on their survival variability post challenge. Using the survival data, high (15%) and less (15%) susceptible cohorts were established for selected candidate genes genotyping by PCR-Length polymorphism (PCR-LP) and Sanger sequencing.

(i) Polymorphism of chicken Mx gene G2032A SNP and survival variability

An allele A is considered resistant, whereas an allele G is considered susceptible, and likewise chickens with genotype AA are considered resistant, whereas birds with genotype GG are considered susceptible (Ko *et al*., 2002, 2004). The frequency of allele A was higher compared to the frequency of allele G in all chicken breeds. The observed high frequency of allele A is in agreement with the previous reports (Li *et al*., 2006; Pagala *et al*., 2013). For example, Indian native chicken, Aseel and Kadaknath show allele A frequency of 0.75 and 0.63, respectively (Ramasamy *et al*., 2017). The highly skewed frequency of A allele may be

explained by environmental selection pressure like a persistent exposure to infectious diseases.

Genotypes AA and AG of chicken Mx gene G2032A SNP were less susceptible compared to genotype GG. Pagala *et al*. (2013) reported similar findings upon challenging adult chickens with NDV where genotypes AA and AG of Tolaki chickens had higher resistance against NDV infection. Less susceptibility of AA and AG genotypes may be conferred by the presence of resistant A allele; whereas, the high susceptibility of GG genotypes may be due to the presence of susceptible G allele. Chicken Mx gene G2032A polymorphism resulting in a substitution of serine with asparagine at position 631 of the chicken *Mx* protein. The change has been demonstrated to influence the antiviral activity of the *Mx* protein (Ko *et al*., 2002).

(ii) Polymorphism of Chicken Mx gene promoter and survival variability

For the first time, the findings demonstrated the association of the promoter region of chicken Mx1 gene polymorphisms with chicken embryos susceptibility to virulent NDV infection. At genotypic level, SNP4 was associated ($P < 0.05$) with phenotype; where at haplotype level, haplotype group ACGC was associated ($P < 0.05$) with the phenotype. The SNP4 is G>A mutation, which was detected at position 194^{th} corresponding to 1 to 284 positions along with the amplified product length polymorphisms for 5' to 3'. A mutation, SNP4, was within AGTTTCGTTTCT motif of ISRE, and the SNP was previously reported in the same position (Li *et al*., 2007). The ISRE plays a crucial role in IFN inducibility of chicken Mx1 gene (Schumacher *et al*., 1994). Results of the present study demonstrated association (LR, 6.97; $P = 0.03$) of genotype GG of SNP4 with less susceptibility of chicken embryos to virulent NDV infection. The genotype GG frequency of 0.7 in the less susceptible group was higher as compared to genotype GG frequency of 0.63 in the high susceptible group. Likewise, although it was not statistically significant, allele G had a higher frequency (0.85) in the less susceptible group as compared with allele A frequency (0.76) in the high susceptible group. Also, allele A had high frequency (0.24) in the high susceptible group as compared with allele A frequency (0.15) in the less susceptible group. It can be said that mutation of G>A is associated with susceptibility of chicken embryos to virulent NDV infection, whereas allele G is associated with resistance of the same trait. The mechanisms underlying this observation remain to be elucidated; however, it may be that the SNP4 G>A mutation alters the functionality of ISRE in IFN inducibility of chicken Mx gene thereby resulting in less expression of the gene, which has been demonstrated to play role in antiviral function.

SNP tags can generate similar phenotypic information that can be obtained by individual SNP (Martin *et al*., 2000). Analysis of SNP haplotypes is effective and less expensive. In the present study, four haplotypes were generated using SNPs that were in LD and were tested for association with chicken embryos susceptibility to virulent NDV challenge. Haplotype group ACGC was significantly (OR, 9.8; 95%CI, 1.06 – 79.43; P = 0.042) associated with chicken embryos susceptibility to virulent NDV infection. The frequency (0.1) of haplotype group ACGC was high in the less susceptible group compared to the high susceptible group, which had a frequency of 0.01. The haplotype group demonstrated to have a protective effect upon chicken embryo challenge with virulent NDV.

(iii) Polymorphisms of LEI0258 microsatellite marker and survival variability

Genetic polymorphism of LEIO258 microsatellite marker and its relationship with chicken embryos survival variability following challenge with virulent NDV was investigated. As described by Fulton *et al*. (2006); two levels of polymorphisms were detected: two repeats: R13 (ATGTCTTCTTTCT) and R12 (TTCCTTCTTTCT, and SNPs and indels in the upstream and downstream flanking sequences. Most of the SNPs and Indels observed in the present study were as previous described by Chazara *et al*. (2013) with the exception that the additional SNP that was observed at position 13 bp downstream flanking sequence was not observed in this study. An additional repeat at position 2 bp upstream of flanking sequences that were not reported by Fulton *et al*. (2006) was also detected in the present study as well. A total of nine SNPs and Indels were detected. Three SNPs and two Indels were detected in the downstream flanking sequences. The SNPs in the downstream flanking sequence were found at positions 3, 37 and 44 pb compared to positions 3, 13, 36 and 43 bp as reported by Chazara *et al*. (2013). Largest deletion (ATTTTGAG) at positions 21-28 bp in the downstream flanking sequences was detected, which is an agreement with the previously report (Chazara *et al*., 2013). Furthermore, the observed correlate between the pattern of Indels and SNPs and allele sizes suggests that knowledge of either of SNPs and/or Indels positions can be used to predict the other, and could be used to predict LEI0258 marker allelic sizes.

The appearance (3 to 13 times) of R12 repeats was very high as compared to that of R13 repeat, which was 1 to 5 times. The higher appearance of R12 has been observed in the previous studies as well (Fulton *et al*., 2006; Chazara *et al*., 2013; Mwambene *et al*., 2019). Also, the mean number of alleles (7) observed in the present study is lower than mean

number of alleles reported in the previously studies (Fulton *et al*., 2006; Lwelamira *et al*., 2008; Schou *et al*., 2010). For instance, in the study that was conducted in Tanzania, the mean number of alleles was 22 and 23 for Kuchi and Medium, respectively (Lwelamira *et al*., 2008). Discrepancy in the mean number of alleles observed maybe explained by differences in sample size and chicken populations involved in the studies. In the present study, selective genotyping was employed where representative samples from high (15%) and less (15%) susceptible chicken embryos were genotyped.

The relationship between genetic polymorphism of LEIO258 microsatellite marker and chicken embryos survival variability following challenge with virulent NDV was also evaluated by the construction of Neighbor-joining unrooted phylogenetic trees to visualize clustering of LEIO258 marker alleles, and also by testing association between MAGs and susceptibility. From the phylogenetic analysis, it is evident that clustering of LEIO258 marker alleles was based on the levels of chicken embryos variation in susceptibility to virulent NDV challenge. LEI0258 alleles from the same cohort (high or less susceptible) tend to cluster together. Clustering of LEI0258 marker alleles based on chicken phenotypes and geographic origin has been observed in previous studies (Fulton *et al*., 2006; Chazara *et al*., 2013). For instance, in a study that was conducted to investigate genetic diversity and relatedness using LEI0258 in chicken breeds from Africa, Asian and Europe, it was observed that chickens from the same geographical location clustered together (Chazara *et al*., 2013). Results of the present study may be suggesting that some LEI0258 marker genetic polymorphisms apart from allelic size (bp) may be linked with chicken MHC–B haplotypes that confer variability in resistance or susceptibility to infections.

However, upon testing of MAGs with chicken embryos variation in susceptibility to virulent NDV challenge, the association was not established with any of the selected MAG ($P > 0.05$). This is in contrast with the previous observation by Lwelamira *et al*. (2008), who reported a positive association between chicken antibody responses against NDV and LEI0258 marker allele 205 bp. The difference observed in the present study may be explained in twofold: first, the difference in phenotypes that were investigated between the studies. In the present study the phenotype was death time of chicken embryos upon infection with virulent NDV, whereas in the previous study, the phenotype was chicken antibody titers following vaccination against NDV; second, the difference in the alleles involved between studies. Furthermore, inconsistency between clustering of LEIO258 alleles based on susceptibility

and lack of association between selected MAGs and susceptibility observed in the present study may be suggesting that other polymorphisms like SNPs and Indels within LEI0258 may be associated with chicken embryos variation in susceptibility to virulent NDV challenge, and maybe the clustering observed in the present study was due to SNPs and Indels within the flanking region of two repeats (R13 and R12) of the LEI0258 microsatellite marker.

Assessment of chicken variations in susceptibility to infections by challenging adult chickens is expensive and prone to confounding factors (Schilling *et al*., 2018). Alternatively, using chicken embryos is less expensive and less exposed to confounding variables because of the eggshell environment (Schilling *et al*., 2018). Chicken embryos (CEs), were used previously to investigated the transcriptional responses of innate immune genes to NDV infection, and the same results were replicated in chicks (Wang *et al*., 2012; Schilling *et al*., 2018). Also, results of embryos and adult chicken were comparable in the previous study (Wang *et al*., 2012) suggesting that the present findings could be replicated in adult chicken.

CHAPTER FIVE

CONCLUSION AND RECOMMENDATIONS

5.1. Conclusion

Chicken remains a dominant poultry species raised by the majority of poor rural households in developing countries of SSA as a chief source of high-quality meat and egg protein and for income generation. However, chicken productivity under backyard settings primarily compromised by diseases, particularly ND. Newcastle disease control and prevention by vaccination and institution of bio-security measures are less effective and plausible in backyard production systems. Genetic selection of chicken genotypes that are less susceptible against NDV is a promising option.

Findings demonstrated higher antibody titres against NDV in kuroiler and local chickens as compared to broiler chickens raised under the same environmental conditions. Overall, the finding contributes to ongoing work in understanding chicken immune responses against NDV and informs breeding programs designed for developing chickens that have increased resistance to NDV.

Furthermore, a combination of candidate gene and selective genotyping approaches were deployed to investigate polymorphisms of selected candidate genes/marker (Mx gene and LEI0258 microsatellite marker) and association with chicken embryos variation in susceptibility to virulent NDV challenge. Polymorphisms of selected candidate genes were associated with chicken embryos variation in susceptibility to virulent NDV challenge. Polymorphisms of chicken Mx G2032A SNP and chicken Mx gene promoter were associated with the phenotype. Specifically, for the first time, the present research demonstrated SNP4 G > A mutation at position 194th of the amplified region (284 bp) of chicken Mx gene promote to be significantly associated with chicken embryos variation in susceptibility to virulent NDV challenge. Single nucleotide change from G to A predisposes chickens to virulent NDV challenge, which suggests allele G to be resistant against NDV. More importantly, haplotype ACGC that was generated by a combination of SNP1, SNP2, SNP4, and SNP5 demonstrated to have a protective effect against virulent NDV in chicken embryos. This information provides a platform for further studies like linking with the chicken Mx gene expression profiles.

Also, clustering of LEIO258 marker alleles in phylogenetic trees was based on the levels of chicken embryos variation in susceptibility to virulent NDV challenge. Interestingly, the LEI0258 marker alleles from the same cohort (high or less susceptible) seem to cluster together. Result suggests that some LEI0258 marker genetic polymorphisms apart from LEI0258 marker allelic size (bp) may be linked with chicken MHC–B haplotypes that confer chickens variability in resistance or susceptibility to infections. The assertion was underscored by lack of association between selected MAGs and chicken embryos variation in susceptibility to virulent NDV challenge. Furthermore, inconsistency between clustering of LEIO258 alleles based on susceptibility and lack of association between selected MAGs and susceptibility observed in the present study maybe suggesting that other polymorphisms apart from LEI0258 marker allelic size like SNPs and Indels within flanking regions of two LEI0258 repeats (R13 and R12) may be linked with chicken embryos variation in susceptibility to virulent NDV challenge.

5.2. Recommendations

Further studies to investigate the effect of SNP and haplotype that was associated with chicken embryos variations in susceptibility to virulent NDV challenge on chicken Mx gene expression profile are highly recommended.

Studies to investigate influence of differential expression profiles of chicken Mx gene due to SNP and haplotype observed in the present study on expression profiles of other genes that have been linked with chicken susceptibility to viral infection are highly recommended, because disease susceptibility is a polygenic trait, which is controlled by many genes and factors.

Studies on the role of polymorphisms apart from LEI0258 marker allelic size like SNPs and Indels within flanking regions of two LEI0258 repeats (R13 and R12) on chicken embryos variation in susceptibility to virulent NDV challenge are recommended.

Finally, conservation of genetic diversity of the outbred chicken population is highly recommended considering the unprecedented effect of emerging and re-emerging infectious disease agents in chicken population. Establishment of institutions within respective ministries in African countries in particular, responsible for coordination of animal genetic resource conservation activities and mobilization of resources for the same is recommended.

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APPENDICES

Appendix 1: Broilers, kuroilers and local chicken weekly body weight raw data that were used in the statistical analysis

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
201	1	1	0	1	524	902	1435	1577	2185	2527	2915
203	1	0	0	1	416	716	1144	1439	2092	2335	2418
204	1	0	0	1	408	741	1203	1584	1635	1962	2269
206	1	1	0	1	413	713	1099	1285	2254	2650	3059
214	1	1	0	1	476	830	1308	1596	2135	2457	2875
218	1	0	0	1	536	940	1418	1559	1980	2310	2724
228	1	1	0	1	517	923	1379	1433	2197	2579	2967
234	1	1	0	1	529	920	1487	1702	2094	2395	2680
238	1	1	0	1	459	791	1244	1695	2249	2677	2878
239	1	0	0	1	531	918	1408	1601	2113	2491	2934
241	1	0	0	1	507	941	1448	1424	2059	2409	2786
247	1	1	0	1	539	925	1485	1639	2310	2839	3449
248	1	0	0	1	479	874	1384	1714	1957	2364	3000
256	1	0	0	1	503	900	1446	1572	1884	2355	2690
258	1	0	0	1	472	807	1222	1575	2265	2672	3176
259	1	0	0	1	488	860	1260	1396	1804	2152	2514
262	1	1	0	1	502	885	1397	1410	2011	2553	2934
266	1	0	0	1	501	871	1293	1684	1897	2335	2788
272	1	0	0	1	458	868	1434	1705	1849	2291	2670
202	1	1	1	1	484	869	1337	1394	1867	2293	2758
273	1	1	1	1	517	875	1377	1743	2483	3041	3343
216	1	0	1	1	555	980	1562	1302	1979	2289	2679
225	1	1	1	1	475	931	1417	1712	2359	2855	3115
231	1	0	1	1	475	880	1399	1451	2046	2443	2632
257	1	0	1	1	493	842	1296	1169	1825	2228	2712
235	1	1	1	1	539	962	1497	1737	2316	2744	3286
236	1	1	1	1	535	918	1452	1725	2343	2704	3260
242	1	1	1	1	491	824	1325	1813	2534	2863	3487
249	1	0	1	1	511	956	1519	1546	2119	2543	2855
255	1	1	1	1	496	886	1364	1680	2244	2794	2800
260	1	0	1	1	506	909	1445	1643	2244	2657	3095
263	1	0	1	1	476	872	1420	1582	1915	2329	2735
264	1	0	1	1	525	920	1405	1477	2044	2352	2785
267	1	1	1	1	506	875	1390	1592	2187	2613	2739
270	1	0	1	1	491	873	1390	1529	2066	2464	2908
269	1	1	5	1	437	763	1184	1732	2254	2573	3188
265	1	1	5	1	515	941	1498	1729	2311	2958	3465
261	1	0	5	1	498	884	1397	1697	2177	2337	2876
254	1	1	5	1	431	819	1219	1566	2044	2479	3118

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
250	1	1	5	1	478	870	1417	1624	2155	2574	3145
245	1	1	5	1	491	875	1439	1800	2377	2694	3057
243	1	0	5	1	526	893	1386	1679	2167	2557	3125
240	1	0	5	1	465	790	1262	1423	1788	2117	2405
232	1	1	5	1	525	961	1476	1832	2355	2713	3345
229	1	0	5	1	475	836	1349	1570	1972	2292	2781
227	1	1	5	1	547	950	1445	1864	2355	2774	3404
226	1	1	5	1	520	881	1338	1365	1962	2681	3231
224	1	0	5	1	564	979	1498	1665	2081	2459	3032
223	1	1	5	1	452	824	1335	1973	2557	2545	2968
221	1	1	5	1	479	845	1246	1652	2086	2443	2549
211	1	1	5	1	473	836	1343	1813	2382	2773	3192
209	1	1	5	1	492	844	1293	1546	1952	2275	2505
205	1	1	7	1	471	853	1364	1549	2143	2600	3045
207	1	0	7	1	499	887	1383	1583	2019	2364	2618
208	1	1	7	1	408	751	1186	1844	2482	2999	3379
210	1	1	7	1	427	718	1097	1599	2087	2452	2798
213	1	1	7	1	485	908	1275	1629	1955	2246	2539
215	1	0	7	1	459	820	1237	1442	1850	2187	2492
217	1	1	7	1	480	843	1295	1692	2231	2541	2815
219	1	0	7	1	467	873	1389	1371	1816	2188	2345
220	1	1	7	1	518	884	1376	1773	2357	2825	3038
230	1	1	7	1	420	757	1188	1813	2443	2885	3266
237	1	0	7	1	496	889	1317	1398	1835	2162	2264
244	1	0	7	1	446	824	1319	1568	2065	2453	2771
246	1	1	7	1	501	840	1279	1668	2180	2571	2934
251	1	0	7	1	498	863	1298	1475	1952	2370	2723
252	1	1	7	1	544	968	1519	1666	2142	2462	2768
253	1	0	7	1	528	934	1421	1498	1933	2192	2546
268	1	1	7	1	483	856	1276	1786	2283	2717	3149
441	1	0	0	2	133	413	856	1162	1526	1846	1850
274	1	0	0	2	203	420	582	1130	1379	1696	1892
277	1	0	0	2	227	416	492	1060	1412	1749	2023
296	1	0	0	2	221	420	796	1158	1479	1685	1875
420	1	1	0	2	186	490	499	1310	1659	1947	2287
417	1	1	0	2	186	446	958	1360	1615	1486	2215
285	1	0	0	2	212	481	885	1104	1364	1650	1714
443	1	0	0	2	191	440	923	871	1107	1314	1484
415	1	0	1	2	204	399	905	899	958	1148	1298
432	1	0	1	2	197	409	867	1494	1812	1894	1751
290	1	1	1	2	197	405	870	1450	1883	3417	2917
449	1	0	1	2	235	380	839	1115	1375	1721	2011
404	1	0	1	2	229	365	1069	723	876	1096	1234

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
427	1	0	1	2	206	396	982	1059	1379	1839	2138
424	1	0	1	2	205	377	713	1227	1551	1924	2003
282	1	0	1	2	188	482	817	1178	1400	1827	2036
287	1	0	1	2	222	445	815	987	1243	1487	1603
408	1	0	1	2	200	385	692	1172	1463	1885	2101
423	1	1	1	2	166	470	977	1132	1589	1969	2106
275	1	0	1	2	203	422	844	1005	1259	1655	1852
405	1	0	1	2	218	416	874	1282	1477	1951	2056
450	1	0	1	2	225	400	796	1216	1476	1889	2239
401	1	1	0	2	210	433	847	1350	1729	2347	2905
278	1	0	5	2	186	384	815	1027	1385	1771	1957
410	1	0	5	2	181	395	811	1059	1424	1797	2090
279	1	1	5	2	209	400	763	1341	1769	1629	2769
439	1	0	5	2	191	390	804	1149	1557	1792	2356
447	1	0	5	2	225	354	777	1128	1450	1632	1932
411	1	1	5	2	218	387	873	1172	1406	1962	2279
442	1	0	5	2	191	331	917	1247	1529	1925	2265
440	1	1	5	2	193	364	973	1160	1528	1786	2288
407	1	0	5	2	213	399	783	1025	1331	1613	1714
402	1	0	5	2	155	344	778	971	1188	1468	1722
436	1	1	5	2	251	444	767	1182	1415	1484	1687
425	1	1	5	2	160	434	676	1266	1499	1911	2024
288	1	1	7	2	170	422	766	1103	1401	1662	1642
294	1	0	7	2	219	494	706	797	1063	1376	1607
434	1	1	7	2	232	475	766	1360	1731	2165	2551
412	1	0	7	2	172	353	772	1170	1531	1906	2060
416	1	0	7	2	186	344	952	1195	1522	1793	2353
293	1	1	7	2	182	390	782	832	1106	1502	1718
430	1	0	7	2	177	413	659	1285	1422	1746	2076
426	1	1	7	2	181	513	644	1232	1601	2076	2343
421	1	0	7	2	223	424	849	1286	1217	1332	1450
409	1	1	7	2	181	394	876	1080	1511	1992	2324
413	1	1	7	2	197	430	919	1402	1885	2118	2672
286	1	1	7	2	176	282	817	1121	1432	1836	2026
429	1	0	7	2	196	331	624	1274	1620	1901	2246
438	1	0	7	2	186	366	732	1267	1590	1950	2224
280	1	0	7	2	118	364	678	1206	1474	1901	2061
297	1	1	7	2	177	416	494	786	1058	1478	1619
406	1	1	7	2	194	463	727	1158	1540	2074	2459
627	2	1	0	1	165	242	444	574	718	900	1116
603	2	0	0	1	140	360	502	471	598	740	879
610	2	1	0	1	176	254	347	545	716	856	1061
620	2	0	0	1	234	333	478	511	660	778	953

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
622	2	1	0	1	251	387	548	762	1000	1207	1491
624	2	1	0	1	225	332	473	727	925	1115	1370
626	2	0	0	1	191	306	451	661	845	990	1179
633	2	0	0	1	225	334	516	515	663	783	934
634	2	0	0	1	220	326	477	683	861	1000	1198
635	2	0	0	1	192	292	412	548	688	808	990
638	2	0	0	1	153	220	319	468	596	695	847
639	2	0	0	1	182	277	392	526	688	843	1055
642	2	0	0	1	251	375	552	519	681	795	958
650	2	1	0	1	179	272	397	695	877	1036	1264
657	2	0	0	1	176	262	383	407	528	625	759
660	2	1	0	1	178	256	383	579	726	848	1036
662	2	0	0	1	180	257	370	510	678	814	1003
663	2	0	0	1	190	281	394	531	649	810	984
668	2	1	0	1	180	263	377	719	964	1162	1425
669	2	0	0	1	193	281	397	624	802	919	1110
604	2	0	2	1	241	361	536	623	763	920	1091
609	2	1	2	1	556	375	568	628	837	1051	1310
614	2	0	2	1	188	266	400	526	675	832	1036
615	2	1	2	1	247	368	516	515	661	810	980
617	2	0	2	1	227	326	466	474	579	694	819
618	2	1	2	1	229	345	4927	539	682	845	1035
619	2	1	2	1	197	297	416	501	649	790	976
628	2	0	2	1	191	258	379	505	626	754	893
636	2	0	2	1	259	392	572	449	562	678	818
644	2	0	2	1	248	381	536	552	713	849	1022
648	2	1	2	1	231	346	490	569	742	921	1150
649	2	1	2	1	179	256	357	864	1099	1329	1577
652	2	1	2	1	196	279	397	669	850	1057	1321
658	2	0	2	1	241	348	512	596	745	891	1052
659	2	1	2	1	197	291	412	533	684	843	1021
667	2	0	2	1	166	236	336	558	685	809	966
605	2	0	6	1	190	295	431	736	900	1067	1272
607	2	1	6	1	157	248	346	634	809	981	1222
613	2	1	6	1	177	268	391	633	736	875	1037
623	2	1	6	1	180	275	392	535	694	840	1075
625	2	1	6	1	183	259	373	615	774	920	1116
630	2	1	6	1	198	268	382	723	907	1083	1223
637	2	1	6	1	196	296	419	596	732	905	1116
640	2	0	6	1	198	304	434	539	685	803	1011
644	2	1	6	1	164	268	415	597	743	897	1104
647	2	0	6	1	258	367	538	429	527	652	821
653	2	1	6	1	152	2290	324	594	729	914	1198

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
654	2	0	6	1	194	278	416	538	662	800	960
655	2	0	6	1	290	446	665	549	668	799	967
661	2	1	6	1	233	358	537	642	809	1006	1241
664	2	1	6	1	209	306	423	649	829	993	1251
666	2	1	6	1	240	360	513	561	852	1004	1265
670	2	1	6	1	211	305	430	463	584	704	876
673	2	0	9	1	189	269	390	445	572	700	756
602	2	1	9	1	206	292	413	712	869	1056	1271
606	2	0	9	1	217	329	462	609	757	910	1029
608	2	1	9	1	154	213	299	711	902	1109	1241
611	2	0	9	1	202	302	450	434	549	677	782
612	2	0	9	1	186	273	392	530	637	773	892
616	2	0	9	1	201	310	439	524	681	820	986
621	2	1	9	1	212	328	467	704	890	1096	1255
629	2	1	9	1	176	267	377	760	947	1147	1311
631	2	0	9	1	179	260	386	627	754	877	993
632	2	0	9	1	222	326	504	463	592	718	826
641	2	0	9	1	172	326	340	506	623	749	882
645	2	1	9	1	227	494	494	598	764	940	1096
646	2	0	9	1	200	300	439	740	915	1107	1273
651	2	1	9	1	120	353	523	556	707	877	1042
656	2	1	9	1	234	341	485	597	745	892	1021
665	2	0	9	1	157	233	334	429	524	624	703
690	2	0	0	2	45	122	255	280	376	492	500
714	2	1	0	2	33	81	51	277	367	519	599
720	2	1	0	2	26	86	486	568	600	684	813
728	2	1	0	2	51	107	163	281	376	434	627
730	2	0	0	2	120	69	200	198	281	357	496
727	2	1	0	2	78	80	356	357	442	511	707
677	2	1	0	2	41	161	392	337	436	572	659
739	2	0	0	2	45	52	270	326	422	554	682
735	2	0	0	2	42	99	230	248	287	388	439
723	2	1	0	2	85	144	263	386	498	594	679
680	2	1	0	2	46	58	205	277	373	499	604
738	2	0	0	2	156	125	91	295	384	517	897
745	2	0	0	2	48	99	231	388	437	535	598
688	2	0	2	2	93	158	377	339	371	443	519
715	2	1	2	2	44	123	236	341	454	513	612
721	2	0	2	2	85	142	211	368	533	750	919
689	2	1	2	2	60	210	223	267	384	465	550
694	2	0	2	2	89	77	173	301	380	458	546
710	2	1	2	2	50	91	283	410	546	678	808
712	2	1	2	2	68	113	306	430	488	600	712

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
713	2	0	2	2	57	88	144	177	202	245	264
729	2	0	2	2	72	146	71	420	578	734	905
681	2	0	2	2	83	102	309	269	340	380	438
701	2	1	2	2	66	102	186	795	962	1200	1472
704	2	0	2	2	54	74	149	463	565	666	707
732	2	0	2	2	83	170	289	296	370	460	479
683	2	0	2	2	82	169	273	351	495	616	626
726	2	1	2	2	56	105	99	390	470	573	674
711	2	1	2	2	72	84	125	426	557	634	717
731	2	1	6	2	73	93	211	340	454	500	557
722	2	0	6	2	55	85	273	227	316	377	469
716	2	1	6	2	93	135	334	430	528	719	793
740	2	0	6	2	68	180	245	279	396	515	638
744	2	0	6	2	62	159	223	220	283	354	444
679	2	0	6	2	70	134	209	282	383	465	479
737	2	0	6	2	54	113	117	352	453	562	536
709	2	0	6	2	54	117	293	221	295	389	499
750	2	1	6	2	86	110	248	486	639	830	920
717	2	1	6	2	58	178	133	304	308	454	625
702	2	1	6	2	63	177	174	428	467	620	684
749	2	0	6	2	68	52	118	240	303	379	417
741	2	0	6	2	49	190	199	172	299	434	529
747	2	1	9	2	47	210	196	212	282	367	429
678	2	1	9	2	55	176	245	299	407	500	623
706	2	0	9	2	104	76	164	258	343	407	440
682	2	1	9	2	49	68	156	735	918	1270	1630
693	2	1	9	2	53	127	192	303	453	579	729
708	2	1	9	2	93	160	198	463	576	710	802
703	2	1	9	2	57	72	150	130	610	723	895
707	2	0	9	2	50	131	90	582	732	868	1023
719	2	0	9	2	50	87	100	548	721	866	877
685	2	0	9	2	47	71	200	314	429	618	476
687	2	0	9	2	89	101	193	481	616	705	843
684	2	0	9	2	50	98	283	238	324	380	407
692	2	1	9	2	52	81	125	453	610	695	642
676	2	1	9	2	96	155	288	301	422	531	626
675	2	1	9	2	90	171	134	247	332	465	551
1015	3	1	0	1	254	373	578	768	1043	1260	1542
1002	3	0	0	1	131	194	287	395	501	615	729
1007	3	1	0	1	235	322	502	736	990	1182	1462
1008	3	1	0	1	212	296	403	734	948	1140	1431
1014	3	0	0	1	217	213	475	599	780	932	1131
1016	3	0	0	1	199	306	431	480	597	715	887

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
1018	3	0	0	1	259	3840	595	465	611	725	903
1019	3	0	0	1	245	352	549	379	480	565	680
1021	3	0	0	1	140	193	267	584	761	924	1074
1022	3	0	0	1	167	233	324	551	716	859	1029
1034	3	0	0	1	127	183	267	467	608	722	871
1035	3	1	0	1	193	276	414	615	788	944	1182
1036	3	0	0	1	221	341	516	729	910	1071	1278
1042	3	0	0	1	217	324	462	464	600	699	882
1048	3	1	0	1	150	368	577	504	639	747	919
1056	3	1	0	1	171	239	355	681	888	1062	1302
1059	3	0	0	1	223	325	505	504	654	769	826
1060	3	1	0	1	173	233	356	617	803	964	1199
1068	3	0	0	1	140	198	288	383	861	994	1189
1071	3	1	0	1	260	362	546	732	930	1135	1434
1011	3	1	3	1	216	322	461	350	462	579	760
1025	3	0	3	1	192	276	406	405	533	662	849
1027	3	0	3	1	204	299	414	578	778	981	1227
1033	3	1	3	1	227	333	512	637	834	1035	1280
1038	3	0	3	1	136	190	294	728	920	1142	1430
1039	3	1	3	1	219	290	424	523	694	841	1120
1040	3	0	3	1	197	281	440	367	480	602	747
1047	3	0	3	1	221	323	489	626	852	1060	1322
1049	3	1	3	1	260	368	547	483	634	782	980
1050	3	0	3	1	253	390	574	567	728	882	1090
1052	3	0	3	1	149	215	303	556	722	892	1093
1058	3	1	3	1	223	324	495	612	822	1043	1312
1062	3	1	3	1	157	232	349	616	792	943	1180
1065	3	0	3	1	218	327	479	403	526	656	821
1069	3	1	3	1	268	383	546	517	673	815	1023
1006	3	0	4	1	138	204	297	571	698	824	948
1009	3	1	4	1	272	377	590	370	487	525	675
1010	3	0	4	1	179	251	395	430	536	590	710
1017	3	1	4	1	141	198	274	665	864	1061	728
1020	3	1	4	1	162	237	343	734	936	1168	1343
1023	3	1	4	1	180	239	351	562	715	885	873
1024	3	0	4	1	150	418	644	665	821	956	1162
1030	3	1	4	1	146	201	306	732	942	964	1219
1037	3	1	4	1	250	220	294	395	507	639	728
1041	3	1	4	1	152	224	325	457	562	685	765
1043	3	1	4	1	195	292	468	836	1042	1270	1497
1045	3	0	4	1	184	273	388	395	492	612	699
1046	3	0	4	1	199	263	389	430	542	665	770
1055	3	0	4	1	218	301	446	489	629	778	845

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
1063	3	0	4	1	230	338	514	549	689	831	937
1066	3	1	4	1	215	301	435	790	979	1221	1403
1070	3	0	4	1	179	261	387	445	556	611	810
1003	3	0	8	1	174	240	354	663	830	1011	1143
1004	3	0	8	1	185	251	375	547	669	824	933
1005	3	0	8	1	263	373	558	609	769	927	1020
1012	3	0	8	1	332	492	723	553	698	863	1050
1013	3	0	8	1	241	354	476	686	823	1018	1187
1026	3	0	8	1	184	265	391	557	693	858	979
1028	3	0	8	1	220	300	456	651	805	1034	1170
1029	3	0	8	1	160	235	350	717	882	1067	1253
1031	3	0	8	1	217	322	470	416	520	654	686
1044	3	0	8	1	178	253	367	422	533	713	779
1051	3	1	8	1	150		305	649	809	1011	1093
1053	3	1	8	1	281	403	610	540	678	827	945
1057	3	0	8	1	265	386	596	945	1197	1456	1675
1061	3	0	8	1	143	361	539	475	615	767	829
1064	3	0	8	1	183	251	385	491	608	750	811
1067	3	1	8	1	179	251	366	796	1010	1240	1323
565	3	0	0	2	104	49	149	326	392	496	652
553	3	0	0	2	66	124	263	425	567	680	863
585	3	1	0	2	53	183	293	665	869	901	1147
574	3	0	0	2	54	107	101	232	307	395	511
583	3	1	0	2	47	127	94	284	384	493	663
594	3	0	0	2	55	113	222	306	423	494	707
587	3	1	3	2	74	66	443	376	518	656	793
570	3	1	3	2	50	78	375	271	383	513	533
578	3	0	3	2	64	214	187	145	196	237	278
599	3	1	3	2	45	161	219	391	591	784	912
595	3	0	3	2	58	108	230	186	227	281	383
580	3	0	3	2	94	133	218	216	310	336	434
555	3	0	3	2	50	79	156	300	430	540	600
575	3	1	3	2	90	122	227	345	479	555	647
552	3	0	3	2	64	170	199	167	274	405	465
597	3	0	3	2	95	159	221	379	506	574	682
592	3	1	3	2	75	139	219	290	386	504	552
584	3	0	3	2	59	80	145	306	424	529	598
551	3	0	3	2	44	101	154	314	417	450	541
567	3	0	4	2	86	93	191	261	360	448	521
563	3	1	4	2	59	125	138	405	542	653	764
572	3	0	4	2	46	90	210	236	340	406	449
576	3	0	4	2	43	92	206	187	249	324	410
579	3	1	4	2	37	133	197	358	523	679	825

TGN	CKN	SEX	PEN	BN	WK1	WK2	WK3	WK4	WK5	WK6	WK7
598	3	0	4	2	62	73	282	120	148	191	219
573	3	1	4	2	46	185	211	554	764	1001	985
586	3	1	4	2	41	77	236	302	416	529	522
571	3	0	4	2	74	80	188	259	357	453	441
593	3	0	4	2	47	111	154	413	539	581	692
600	3	0	8	2	71	94	213	315	423	486	444
554	3	0	8	2	50	84	165	331	469	605	676
561	3	0	8	2	55	108	194	319	475	576	741
564	3	0	8	2	48	53	145	444	551	680	815
560	3	0	8	2	59	109	171	571	706	877	575
557	3	0	8	2	61	97	198	354	416	531	627
559	3	0	8	2	56	100	169	320	457	626	724
577	3	1	8	2	71	68	245	226	358	450	629
581	3	0	8	2	50	100	221	321	412	483	575
588	3	0	8	2	60	91	197	240	320	342	452
569	3	0	8	2	58	83	172	264	307	369	405
562	3	0	8	2	66	97	297	222	295	403	527
568	3	0	8	2	59	82	214	340	408	502	657

Legend: BN is batch number; CKN is chicken type (1 = broiler, 2 = kuroiler, 3 = local chicken); TGN is tag number; PEN 0 was for control group (unvaccinated); W1, W2, W3, W4, W5, W6, and W7=, body weight in grams (g) at 1, 2, 3, 4, 5, 6 and 7 weeks of age; g is grams.

Appendix 2: Broilers, kuroilers and local chicken antibody titre raw data that were used in the statistical analysis

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
201	1	1	0	1	2.423	2.192
203	1	0	0	1	2.400	2.163
204	1	0	0	1	2.678	2.153
206	1	1	0	1	1.696	2.025
214	1	1	0	1	2.596	2.173
218	1	0	0	1	2.395	1.122
228	1	1	0	1	2.678	2.111
234	1	1	0	1	2.111	2.479
238	1	1	0	1	2.346	2.183
239	1	0	0	1	2.479	2.111
241	1	0	0	1	2.183	2.346
247	1	1	0	1	2.678	2.678
248	1	0	0	1	2.163	2.025
256	1	0	0	1	2.153	2.423
258	1	0	0	1	2.025	2.268
259	1	0	0	1	2.173	2.011
262	1	1	0	1	1.122	2.444
266	1	0	0	1	2.484	2.423
272	1	0	0	1	2.678	1.997
202	1	1	1	1	4.096	4.330
273	1	1	1	1	3.881	4.094
216	1	0	1	1	3.706	4.295
225	1	1	1	1	4.225	4.014
231	1	0	1	1	3.891	4.000
257	1	0	1	1	3.652	4.111
235	1	1	1	1	3.870	4.168
236	1	1	1	1	4.022	4.000
242	1	1	1	1	4.295	4.295
249	1	0	1	1	3.439	4.014
255	1	1	1	1	4.053	4.084
260	1	0	1	1	3.811	4.194
263	1	0	1	1	3.729	4.105
264	1	0	1	1	4.145	4.282
267	1	1	1	1	4.171	4.244
270	1	0	1	1	3.715	4.084
269	1	1	5	1	3.729	4.194
265	1	1	5	1	4.053	4.105
261	1	0	5	1	3.706	3.913
254	1	1	5	1	3.729	4.000
250	1	1	5	1	3.771	4.039
245	1	1	5	1	3.992	4.191

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
243	1	0	5	1	3.890	4.140
240	1	0	5	1	3.736	4.199
232	1	1	5	1	3.992	4.230
229	1	0	5	1	3.364	4.282
227	1	1	5	1	3.597	4.244
226	1	1	5	1	3.706	4.107
224	1	0	5	1	3.954	4.107
223	1	1	5	1	4.202	4.230
221	1	1	5	1	3.692	3.913
211	1	1	5	1	4.200	4.234
209	1	1	5	1	4.131	4.234
205	1	1	7	1	3.706	3.913
207	1	0	7	1	3.954	4.107
208	1	1	7	1	4.202	4.107
210	1	1	7	1	3.656	3.636
213	1	1	7	1	3.954	4.121
215	1	0	7	1	4.202	4.363
217	1	1	7	1	4.004	4.107
219	1	0	7	1	4.004	4.107
220	1	1	7	1	4.065	3.965
230	1	1	7	1	3.992	4.143
237	1	0	7	1	3.692	1.298
244	1	0	7	1	4.200	2.454
246	1	1	7	1	4.008	3.791
251	1	0	7	1	3.721	3.977
252	1	1	7	1	3.484	3.891
253	1	0	7	1	3.656	3.891
268	1	1	7	1	3.954	4.069
441	1	0	0	2	2.298	2.291
274	1	0	0	2	1.900	3.239
277	1	0	0	2	2.353	0.520
296	1	0	0	2	1.882	2.291
420	1	1	0	2	1.423	3.389
417	1	1	0	2	2.298	1.298
285	1	0	0	2	2.260	2.454
443	1	0	0	2	2.326	2.326
415	1	0	1	2	3.389	3.389
432	1	0	1	2	2.268	3.612
290	1	1	1	2	3.792	3.412
449	1	0	1	2	3.534	4.153
404	1	0	1	2	2.846	2.326
427	1	0	1	2	3.054	2.326
424	1	0	1	2	3.272	3.988

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
282	1	0	1	2	3.278	2.887
287	1	0	1	2	3.690	2.794
408	1	0	1	2	3.534	3.509
423	1	1	1	2	2.836	4.053
275	1	0	1	2	3.218	3.292
405	1	0	1	2	3.563	3.901
450	1	0	1	2	3.394	3.903
401	1	1	0	2	3.563	3.544
278	1	0	5	2	2.581	3.265
410	1	0	5	2	3.044	3.681
279	1	1	5	2	3.614	3.615
439	1	0	5	2	3.240	3.583
447	1	0	5	2	3.481	3.824
411	1	1	5	2	3.093	3.681
442	1	0	5	2	2.938	2.801
440	1	1	5	2	3.353	3.681
407	1	0	5	2	3.297	3.292
402	1	0	5	2	4.060	3.901
436	1	1	5	2	3.240	3.583
425	1	1	5	2	3.297	3.544
288	1	1	7	2	3.655	3.265
294	1	0	7	2	3.363	3.544
434	1	1	7	2	3.698	3.265
412	1	0	7	2	2.782	3.432
416	1	0	7	2	1.599	3.892
293	1	1	7	2	3.093	3.589
430	1	0	7	2	3.346	3.681
426	1	1	7	2	2.900	3.539
421	1	0	7	2	2.745	2.916
409	1	1	7	2	2.672	3.798
413	1	1	7	2	2.761	3.681
286	1	1	7	2	3.475	3.550
429	1	0	7	2	3.968	3.213
438	1	0	7	2	3.134	2.817
280	1	0	7	2	3.681	3.751
297	1	1	7	2	2.761	3.069
406	1	1	7	2	2.794	4.184
627	2	1	0	1	2.395	2.439
603	2	0	0	1	1.997	1.997
610	2	1	0	1	1.520	1.553
620	2	0	0	1	2.025	2.312
622	2	1	0	1	2.025	2.312
624	2	1	0	1	1.842	2.928

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
626	2	0	0	1	2.395	2.730
633	2	0	0	1	1.219	1.219
634	2	0	0	1	2.268	1.634
635	2	0	0	1	2.076	2.650
638	2	0	0	1	2.541	2.064
639	2	0	0	1	2.444	2.444
642	2	0	0	1	2.244	2.444
650	2	1	0	1	2.319	1.799
657	2	0	0	1	2.620	0.520
660	2	1	0	1	2.782	2.011
662	2	0	0	1	2.319	1.951
663	2	0	0	1	2.353	2.484
668	2	1	0	1	2.133	2.911
669	2	0	0	1	2.319	2.319
604	2	0	2	1	3.950	4.247
609	2	1	2	1	4.011	4.257
614	2	0	2	1	4.113	4.247
615	2	1	2	1	4.296	4.257
617	2	0	2	1	4.000	4.211
618	2	1	2	1	3.774	4.031
619	2	1	2	1	3.950	3.917
628	2	0	2	1	4.011	4.216
636	2	0	2	1	1.298	4.174
644	2	0	2	1	4.181	4.202
648	2	1	2	1	3.663	4.247
649	2	1	2	1	3.663	4.257
652	2	1	2	1	4.181	4.004
658	2	0	2	1	4.202	4.202
659	2	1	2	1	4.069	4.281
667	2	0	2	1	3.887	4.124
605	2	0	6	1	4.241	4.168
607	2	1	6	1	3.999	4.257
613	2	1	6	1	4.054	4.054
623	2	1	6	1	4.139	4.264
625	2	1	6	1	4.181	4.200
630	2	1	6	1	3.102	4.141
637	2	1	6	1	4.291	4.328
640	2	0	6	1	3.811	4.121
644	2	1	6	1	3.908	4.110
647	2	0	6	1	3.895	3.514
653	2	1	6	1	4.159	4.221
654	2	0	6	1	4.069	3.959
655	2	0	6	1	3.514	3.807

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
661	2	1	6	1	4.284	4.298
664	2	1	6	1	3.877	4.109
666	2	1	6	1	3.508	3.618
670	2	1	6	1	3.247	3.618
673	2	0	9	1	3.844	4.167
602	2	1	9	1	4.041	3.959
606	2	0	9	1	3.768	3.942
608	2	1	9	1	3.887	4.134
611	2	0	9	1	4.141	4.280
612	2	0	9	1	3.877	4.373
616	2	0	9	1	3.575	4.183
621	2	1	9	1	4.114	4.114
629	2	1	9	1	4.259	4.007
631	2	0	9	1	4.122	4.115
632	2	0	9	1	4.229	4.150
641	2	0	9	1	4.317	4.114
645	2	1	9	1	3.751	4.181
646	2	0	9	1	2.925	4.062
651	2	1	9	1	4.058	4.165
656	2	1	9	1	3.945	4.131
665	2	0	9	1	3.921	3.877
690	2	0	0	2	1.696	2.474
714	2	1	0	2	2.418	1.122
720	2	1	0	2	2.502	0.520
728	2	1	0	2	2.423	2.401
730	2	0	0	2	3.157	1.474
727	2	1	0	2	2.064	0.997
677	2	1	0	2	1.696	2.474
739	2	0	0	2	3.139	1.951
735	2	0	0	2	2.423	1.696
723	2	1	0	2	3.157	1.520
680	2	1	0	2	1.666	3.476
738	2	0	0	2	3.107	2.252
745	2	0	0	2	1.696	4.476
688	2	0	2	2	3.009	1.666
715	2	1	2	2	3.770	3.408
721	2	0	2	2	3.271	3.480
689	2	1	2	2	4.112	4.166
694	2	0	2	2	2.866	3.540
710	2	1	2	2	3.346	3.673
712	2	1	2	2	3.721	3.540
713	2	0	2	2	4.029	2.735
729	2	0	2	2	3.654	4.169

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
681	2	0	2	2	3.152	4.083
701	2	1	2	2	3.851	7.079
704	2	0	2	2	3.724	7.027
732	2	0	2	2	4.081	3.821
683	2	0	2	2	4.054	3.652
726	2	1	2	2	3.917	4.083
711	2	1	2	2	3.872	7.079
731	2	1	6	2	4.285	4.003
722	2	0	6	2	3.389	1.951
716	2	1	6	2	6.705	3.875
740	2	0	6	2	3.848	3.394
744	2	0	6	2	3.424	3.795
679	2	0	6	2	3.143	3.875
737	2	0	6	2	3.080	3.708
709	2	0	6	2	3.851	3.958
750	2	1	6	2	3.848	3.967
717	2	1	6	2	2.613	3.688
702	2	1	6	2	3.851	3.618
749	2	0	6	2	4.085	4.321
741	2	0	6	2	3.965	3.756
747	2	1	9	2	3.648	3.989
678	2	1	9	2	3.843	4.170
706	2	0	9	2	3.488	3.663
682	2	1	9	2	3.549	3.498
693	2	1	9	2	3.848	4.185
708	2	1	9	2	3.713	3.663
703	2	1	9	2	3.078	4.119
707	2	0	9	2	4.090	3.629
719	2	0	9	2	4.051	4.028
685	2	0	9	2	3.974	4.252
687	2	0	9	2	3.844	3.809
684	2	0	9	2	3.489	3.988
692	2	1	9	2	3.515	4.067
676	2	1	9	2	3.974	3.964
675	2	1	9	2	3.844	1.122
1015	3	1	0	1	2.444	2.076
1002	3	0	0	1	2.439	2.439
1007	3	1	0	1	2.898	2.312
1008	3	1	0	1	2.268	1.724
1014	3	0	0	1	2.678	2.620
1016	3	0	0	1	2.319	2.978
1018	3	0	0	1	2.559	2.454
1019	3	0	0	1	2.163	2.163

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
1021	3	0	0	1	2.326	2.454
1022	3	0	0	1	2.383	2.650
1034	3	0	0	1	1.634	2.785
1035	3	1	0	1	1.298	0.997
1036	3	0	0	1	2.545	2.371
1042	3	0	0	1	1.634	2.606
1048	3	1	0	1	2.573	2.488
1056	3	1	0	1	1.750	1.634
1059	3	0	0	1	2.383	2.395
1060	3	1	0	1	2.353	1.842
1068	3	0	0	1	2.511	1.298
1071	3	1	0	1	1.842	1.666
1011	3	1	3	1	4.240	4.256
1025	3	0	3	1	4.196	4.333
1027	3	0	3	1	3.684	4.202
1033	3	1	3	1	3.736	4.275
1038	3	0	3	1	4.162	4.095
1039	3	1	3	1	4.238	4.275
1040	3	0	3	1	4.182	4.178
1047	3	0	3	1	3.886	3.976
1049	3	1	3	1	3.790	4.223
1050	3	0	3	1	3.886	3.976
1052	3	0	3	1	4.159	4.182
1058	3	1	3	1	3.668	4.018
1062	3	1	3	1	3.617	4.122
1065	3	0	3	1	4.156	4.334
1069	3	1	3	1	4.136	4.217
1006	3	0	4	1	4.218	4.331
1009	3	1	4	1	4.200	4.122
1010	3	0	4	1	4.200	4.216
1017	3	1	4	1	3.617	4.092
1020	3	1	4	1	3.960	4.231
1023	3	1	4	1	4.201	4.347
1024	3	0	4	1	7.091	4.122
1030	3	1	4	1	2.962	3.287
1037	3	1	4	1	4.008	4.231
1041	3	1	4	1	4.131	4.070
1043	3	1	4	1	3.780	3.894
1045	3	0	4	1	3.804	4.216
1046	3	0	4	1	3.912	4.314
1055	3	0	4	1	3.849	4.236
1063	3	0	4	1	4.076	4.231
1066	3	1	4	1	3.861	3.932

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
1070	3	0	4	1	4.110	4.177
1003	3	0	8	1	3.785	4.078
1004	3	0	8	1	3.606	3.836
1005	3	0	8	1	3.954	4.199
1012	3	0	8	1	3.956	4.199
1013	3	0	8	1	4.021	4.000
1026	3	0	8		2.756	4.101
1028	3	0	8	1	3.914	4.297
1029	3	0	8	1	3.981	4.261
1031	3	0	8	1	3.867	3.711
1044	3	0	8	1	3.350	4.000
1051	3	1	8	1	3.748	4.101
1053	3	1	8	1	2.862	4.347
1057	3	0	8	1	4.214	4.365
1061	3	0	8	1	3.997	3.901
1064	3	0	8	1	3.659	4.302
1067	3	1	8	1	3.376	4.216
565	3	0	0	2	2.637	1.298
553	3	0	0	2	2.449	1.775
585	3	1	0	2	3.111	1.696
574	3	0	0	2	1.775	1.298
583	3	1	0	2	2.637	1.298
594	3	0	0	2	2.268	1.775
587	3	1	3	2	3.386	3.653
570	3	1	3	2	2.581	4.045
578	3	0	3	2	2.806	3.366
599	3	1	3	2	3.972	3.980
595	3	0	3	2	4.051	4.236
580	3	0	3	2	3.924	3.939
555	3	0	3	2	3.735	3.697
575	3	1	3	2	3.394	3.923
552	3	0	3	2	2.778	3.033
597	3	0	3	2	3.932	4.188
592	3	1	3	2	3.802	3.647
584	3	0	3	2	3.294	3.163
551	3	0	3	2	3.406	3.833
567	3	0	4	2	3.802	3.647
563	3	1	4	2	3.762	3.883
572	3	0	4	2	3.840	3.163
576	3	0	4	2	3.197	3.629
579	3	1	4	2	3.647	3.218
598	3	0	4	2	2.100	3.669
573	3	1	4	2	2.959	3.881

TGN	CKN	SEX	PEN	BN	Log10AB10	Log10AB21
586	3	1	4	2	3.434	3.480
571	3	0	4	2	3.373	4.216
593	3	0	4	2	3.686	3.996
600	3	0	8	2	3.267	3.110
554	3	0	8	2	4.068	3.490
561	3	0	8	2	3.737	3.327
564	3	0	8	2	3.191	3.720
560	3	0	8	2	3.373	3.908
557	3	0	8	2	3.686	3.771
559	3	0	8	2	3.553	3.908
577	3	1	8	2	3.073	4.048
581	3	0	8	2	3.214	3.952
588	3	0	8	2	4.090	3.375
569	3	0	8	2	3.537	3.490
562	3	0	8	2	3.045	3.100
568	3	0	8	2	3.722	3.903

Legend: BN is batch number; CKN is chicken type (1 = broiler, 2 = kuroiler, 3 = local chicken); TGN is tag number; PEN 0 was for control group (unvaccinated); log10AB10 is log10 antibody titres at day 10 post vaccination; log10AB21 is log10 antibody titres at day 21 post vaccination.

Appendix 3: Chicken Mx1 gene G2032A SNP genotype frequency raw data

Sample ID	Breed	Susceptibility	Genotype
k60	kuroiler	L	AG
S76	sasso	L	AA
l103	local	H	AG
l110	local	H	AG
l41	local	L	AG
s61	sasso	L	AG
k48	kuroiler	H	AG
l6	local	H	AA
s30	sasso	L	AA
s68	sasso	H	AA
k126	kuroiler	H	AA
s7	sasso	L	AG
k34	kuroiler	L	AG
l5	local	H	AA
s65	sasso	H	AG
k20	kuroiler	L	AA
k112	kuroiler	H	AA
s69	sasso	H	AG
k110	kuroiler	L	AG
l113	local	L	AA
k27	kuroiler	H	AG
l68	local	H	AA
k57	kuroiler	L	AG
s64	sasso	L	AA
s24	sasso	H	AA
l98	local	L	AA
k16	kuroiler	H	AA
l38	local	L	AA
k42	kuroiler	H	AA
l51	local	L	AA
k102	kuroiler	L	AG
l80	local	L	AA
l95	local	L	AA
k38	kuroiler	H	AG
k33	kuroiler	H	AA
s50	sasso	H	AG
k119	kuroiler	H	AA
s52	sasso	L	AG
k55	kuroiler	L	AA
l91	local	L	AA
k1	kuroiler	L	AA

Sample ID	Breed	Susceptibility	Genotype
k103	kuroiler	L	AA
l84	local	H	AA
l24	local	L	AA
s31	sasso	H	AA
s40	sasso	L	AA
k82	kuroiler	L	AA
k50	kuroiler	L	AA
k89	kuroiler	H	GG
l117	local	H	AG
l12	local	L	GG
l35	local	L	GG
l19	local	H	GG
l48	local	H	GG
s34	sasso	L	GG
k59	kuroiler	H	GG
s54	sasso	H	GG
l4	local	L	AA
s33	sasso	H	AG
s47	sasso	L	AG
l8	local	L	AA
k14	kuroiler	L	AG
l78	local	L	AA
l75	local	H	AG
s84	sasso	H	AA
k58	kuroiler	L	AG
k17	kuroiler	H	AG
l59	local	L	AA
l39	local	L	AG
k56	kuroiler	L	AA
k12	kuroiler	L	AA
k22	kuroiler	L	AA
k35	kuroiler	L	AA
l63	local	L	AA
l55	local	L	AG
s59	sasso	H	AA
l23	local	H	AG
l52	local	H	AG
s6	sasso	H	AA
l16	local	L	AG
k7	kuroiler	L	AG
l54	local	H	AG
l3	local	H	AG
k24	kuroiler	L	AA

Sample ID	Breed	Susceptibility	Genotype
l10	local	H	AG
s88	sasso	L	AG
s51	sasso	L	AG
s46	sasso	L	AG
k117	kuroiler	L	AG
k75	kuroiler	H	AG

Legend: K is kuroiler, L is local Tanzanian chicken, and S is Sasso; H is highly susceptible, and L is less susceptible

Appendix 4: Genotypes of five SNPs observed in 284 bp chicken Mx1 gene promoter sequences

Sample ID	Susceptibility	SNP1	SNP2	SNP3	SNP4	SNP5
K1	L	AA	CC	CC	GG	TT
K112	H	AA	CC	CT	GG	TT
K117	L	AA	CC	TT	GG	TT
K119	H	AT	GC	CC	AA	CT
K12	L	AA	CC	CT	GG	CT
K126	H	AA	CC	CC	GG	TT
K14	L	AT	GC	CC	AG	CT
K16	H	AA	CC	CT	GG	TT
K17	H	AA	CC	CT	GG	TT
K20	L	AA	CC	CC	GG	TT
K22	L	AT	GC	CT	AG	CT
K24	L	AA	CC	CC	GG	TT
K27	H	AA	CC	CT	GG	TT
K33	H	AA	CC	CT	GG	TT
K34	L	AA	CC	TT	GG	TT
K38	H	AT	GC	CC	AG	CT
K42	H	AT	GC	CT	AG	CT
K57	L	AA	CC	TT	GG	CT
K58	L	AT	GC	CC	AG	CT
K59	H	AT	GC	CC	AG	CT
K60	L	AA	CC	CT	GG	TT
K7	L	AT	GC	CT	AG	CC
K75	H	AT	GC	CC	AG	CT
K89	H	AT	GC	CT	AG	CT
L103	H	AA	CC	CC	GG	TT
L104	H	AA	CC	TT	GG	TT
L110	L	AA	CC	CC	GG	TT
L113	L	AA	CC	CT	GG	TT
L117	H	AA	CC	CC	GG	TT
L12	L	AA	CC	CC	GG	TT
L16	L	AA	CC	CC	GG	TT
L19	H	AA	CC	CC	GG	TT
L23	H	AT	GC	CC	AG	CT
L24	L	AA	CC	CC	GG	TT
L3	H	AA	CC	CT	GG	TT
L35	L	AA	CC	CC	GG	TT
L38	L	AA	CC	CC	GG	TT
L39	L	AA	CC	CC	GG	TT
L41	L	AA	CC	CC	GG	TT
L4	L	AA	CC	CT	GG	TT
L48	H	AA	CC	CC	GG	TT

Sample ID	Susceptibility	SNP1	SNP2	SNP3	SNP4	SNP5
L5	H	AA	GC	CC	GG	TT
L51	H	AA	CC	CT	GG	TT
L52	H	AA	CC	TT	GG	TT
L54	H	AA	CC	CC	GG	TT
L55	L	AA	CC	CC	GG	TT
L59	L	AA	CC	CC	GG	TT
L6	H	AA	CC	CC	GG	TT
L63	L	AA	CC	CC	GG	TT
L68	H	AA	CC	CC	GG	TT
L75	H	AA	CC	CT	GG	TT
L78	L	AA	CC	CC	GG	TT
L8	L	AT	GC	CC	AG	CT
L80	L	AA	GC	CC	GG	TT
L91	L	AA	CC	CC	GG	TT
L98	L	AA	CC	CC	GG	TT
S24	H	AA	CC	CC	GG	TT
S30	L	AA	CC	CT	GG	CT
S31	H	AT	GC	CT	AG	CT
S33	H	AA	CC	CC	GG	TT
S34	L	AA	CC	CT	GG	TT
S35	L	AA	CC	CT	GG	CT
S40	L	AA	CC	TT	GG	CT
S46	L	AA	CC	CT	GG	TT
S47	L	AA	CC	CT	GG	CT
S50	H	AT	GC	CT	AG	CC
S51	L	AT	GC	CT	AG	CC
S52	L	AT	GC	CC	AG	CT
S54	L	AT	GC	CT	AG	CT
S59	H	TT	GG	CC	AA	CC
S6	H	TT	GG	CC	AA	CC
S61	L	AT	GC	CC	AG	CT
S64	L	AT	GC	CT	AG	CC
S65	H	AA	CC	CC	GG	TT
S68	H	AA	CC	CT	GG	TT
S69	H	AT	GC	CC	AG	CT
S76	L	AT	GC	CT	AG	CC
S84	H	TT	GG	CC	AA	CC
S88	L	AT	GC	CC	AG	CT
k50	L	AA	CC	CT	GG	TT
K121	H	AT	GC	CT	AG	CT
L10	H	AA	CC	CC	GG	TT
S7	L	AA	CC	TT	GG	TT
K103	L	AT	GC	CT	AG	CT

Sample ID	Susceptibility	SNP1	SNP2	SNP3	SNP4	SNP5
K110	L	AT	GC	CC	AG	CT
K55	L	AT	GC	CC	AG	CT
K56	L	AA	CC	CC	GG	TT
K102	L	AA	CC	CT	GG	TT

Legend: K is kuroiler, L is local Tanzanian chicken, and S is Sasso; H is highly susceptible, and L is less susceptible

Appendix 5: LEIO258 marker polymorphisms identified in kuroiler, Sasso, and local Tanzanian chicken

			Upstream							Downstream			
Consensus (bp)	Name	Susceptibility	-32-31 TT/ Δ	-30 G/A	-12 G/A	-2 C/T	R13	R12	3 C/T	21-28 ATTTTGAG/ Δ	31 Δ/A	37 A/T	44 T/A
194	K39	H	-	-	A	-	1	3	-	Δ	A	-	-
194	K7	L	-	-	A	-	1	3	-	Δ	A	-	-
194	K60	L	-	-	A	-	1	3	-	Δ	A	-	-
194	K84	H	-	-	A	-	1	3	-	Δ	A	-	-
194	K121	H	-	-	A	-	1	3	-	Δ	A	-	-
205	L48	H	-	-	-	-	1	4	-	Δ	-	-	-
205	S6	H	-	-	-	-	1	4	-	Δ	-	-	-
205	S61	L	-	-	-	-	1	4	-	Δ	-	-	-
205	S65	H	-	-	-	-	1	4	-	Δ	-	-	-
205	S92	H	-	-	-	-	1	4	T	Δ	-	-	-
205	L35	L	-	-	-	-	1	4	-	Δ	-	-	-
205	S52	L	-	-	-	-	1	4	-	Δ	-	-	-
205	S68	H	-	-	-	-	1	4	-	Δ	-	-	-
205	L19	H	-	-	-	-	1	4	-	-	-	-	-
194	K27	H	-	-	A	-	1	3	-	Δ	A	-	-
217	K75	H	-	-	-	-	1	5	-	Δ	-	-	-
217	K129	L	-	-	-	-	1	5	-	Δ	-	-	-
217	L6	H	-	-	-	-	1	5	-	Δ	-	-	-
217	L12	L	-	-	-	-	1	5	-	Δ	-	-	-
217	L23	H	-	-	-	-	1	5	-	Δ	-	-	-
217	L59	L	-	-	-	T	1	5	-	Δ	-	-	-
217	K112	H	-	-	-	-	1	5	-	-	-	-	-
241	S24	H	-	-	-	-	1	7	-	Δ	-	-	-

			Upstream							Downstream			
Consensus (bp)	Name	Susceptibility	-32-31 TT/ Δ	-30 G/A	-12 G/A	-2 C/T	R13	R12	3 C/T	21-28 ATTTTGAG/ Δ	31 Δ/A	37 A/T	44 T/A
241	S84	H	-	-	-	-	1	7	-	Δ	-	-	-
249	K34	L	-	-	-	-	1	7	-	-	-	-	A
249	S7	L	-	-	-	-	1	7	-	-	-	-	A
249	S46	L	-	-	-	-	1	7	-	-	-	-	A
249	S88	L	-	-	-	-	1	7	-	-	-	-	A
249	K56	L	-	-	-	-	1	7	-	-	-	-	A
205	S31	H	-	-	-	-	1	4	T	Δ	-	-	-
261	S34	L	-	-	-	-	1	8	-	-	-	-	-
205	S47	L	-	-	-	-	1	4	-	Δ	-	-	-
261	L4	L	-	-	-	-	1	8	-	-	-	-	A
261	L10	H	-	-	-	-	1	8	-	-	-	-	A
261	L24	L	-	-	-	-	1	8	-	Δ	-	-	A
261	L16	L	-	-	-	-	1	8	-	-	-	-	A
261	S69	H	-	-	-	-	1	8	-	-	-	-	A
261	K48	H	-	-	-	-	1	8	-	-	-	-	A
261	L5	H	-	-	-	-	1	8	-	-	-	-	A
261	L103	H	-	-	-	-	1	8	-	-	-	-	A
261	S76	L	-	-	-	-	1	8	-	-	-	-	-
217	K16	H	-	-	-	-	1	5	-	Δ	-	-	-
261	L41	L	-	-	-	-	1	8	-	-	A	-	A
261	LT2	H	-	-	-	-	1	8	-	-	-	-	A
261	K57	L	-	-	-		1	8	-	-	-	-	A
261	L95	L	-	-	-	-	1	8	-	-	-	-	A
205	S40	L	-	-	-	T	1	4	T	Δ	-	-	
273	K59	H	-	-	-	-	1	9	-	-	-	-	A

			Upstream							Downstream			
Consensus (bp)	Name	Susceptibility	-32-31 TT/ Δ	-30 G/A	-12 G/A	-2 C/T	R13	R12	3 C/T	21-28 ATTTTGAG/ Δ	31 Δ/A	37 A/T	44 T/A
217	K22	L	-	-	-	-	1	5	-	Δ	-	-	-
194	K42	H	-	-	A	-	1	3	-	Δ	A	-	-
273	K103	L	-	-	-	-	1	9	-	-	-	-	A
261	K110	L	-	-	-	-	1	8	-	-	-	-	A
295	L63	L	Δ	-	-	-	1	11	-	-	-	-	-
307	K89	H	Δ	A	-	-	1	12	-	-	-	-	-
307	L51	H	Δ	A	-	-	1	12	-	-	-	-	-
307	L75	H	-	-	-	-	1	12	-	-	-	-	-
307	L110	H	Δ	A	-	-	1	12	-	-	-	-	-
194	K1	L	-	-	A	-	1	3	-	Δ	A	-	-
307	K80	L	Δ	A	-	-	1	12	-	-	-	-	-
307	L52	H	Δ	A	-	-	1	12	-	-	-	-	-
309	L68	H	-	-	-	-	1	12	-	-	-	T	-
309	K24	L	-	-	-	-	1	12	-	-	-	T	-
309	K50	L	-	-	-	-	1	12	-	-	-	T	-
309	K119	H	-	-	-	-	1	12	-	-	-	T	-
309	K126	H	-	-	-	-	1	12	-	-	-	T	-
309	K102	L	-	-	-	-	1	12	-	-	-	T	-
273	K14	L	-	-	-	T	1	9	-	-	-	-	A
317	K55	L	-	-	-	-	1	9	-	-	-	-	-
273	L3	H	-	-	-	-	1	9	-	-	-	-	-
217	L39	L	-	-	-	T	1	5	-	Δ	-	-	-
452	S59	H	-	-	-	-	4	5	-	-	-	-	-
307	L104	H	Δ	A	-	-	1	12	-	-	-	-	-

			Upstream						Downstream				
Consensus (bp)	**Name**	**Susceptibility**	**-32-31**	**-30**	**-12**	**-2**			**3**	**21-28**	**31**	**37**	**44**
			TT/ Δ	**G/A**	**G/A**	**C/T**	**R13**	**R12**	**C/T**	**ATTTTGAG/ Δ**	**Δ/A**	**A/T**	**T/A**
205	LS4	H	-	-	-	-	1	4	T	Δ	-	-	-
312	L84	H	-	-	-	-	1	9	-	-	-	-	-
312	L38	L	-	-	-	-	1	9	-	-	-	-	-

Legend: "Δ" Defines deletion compared with the reference sequence.

"–" Consistent with the reference sequence