EVALUATION OF INNATE IMMUNITY TO NEWCASTLE DISEASE IN SELECTED FREE-RANGE LOCAL CHICKEN ECOTYPES IN TANZANIA

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EXTENDED ABSTRACT

Newcastle disease (ND) is a major problem in poultry husbandry, causing tremendous losses to farmers in Tanzania. The problem is even bigger in the extensive free-range management system practices for free-range local chickens (FRLC) in rural areas of the country. Practical control of ND in FRLC is mainly by vaccination. The extensive husbandry system exposes FRLC to high risk of ND infection due to interaction with other bird species and contaminated environment. This study focused on the evaluation of ND in FRLC flocks to improve their productivity through selection of ND resistant FRLC ecotypes in Tanzania. A flock of 389 FRLC (324 females and 65 males) of three Tanzanian ecotypes namely; Ching'wekwe, Morogoro-medium and Kuchi were sampled from the coastal, central, northern and lake zones of Tanzania to establish a breeding parent stock for experiments. Each chicken was identified using numbered wing tag to maintain identity between parents and progenies. The chickens were first characterized phenotypically and genotypically. From the parents, morphometric parameters were taken, i.e. body length (BL), neck length (NL), chest girth (CG), shank length (SL) and shank girth (SG) were measured in centimetres (cm) using a tailor's measuring tape. Body weights (BW) were measured in grams (gm) using a 0.01gm sensitive electronic weighing scale. These measurements were analysed using one-way analysis of variance (ANOVA) and compared among the three ecotypes. Differences in traits between ecotypes were considered significant at $p \leq 0.05$. Results from the morphometric analyses showed that the chickens are three distinctly three ecotypes. A total of 1,399 progeny chicks (477 Ching'wekwe, 315 Kuchi, and 607 Morogoro-medium) were produced from the parent stocks in five rounds of incubation and

hatching for use in the determination of immune response traits during infection with ND and also for determination of the population structure using single nucleotide polymorphism (SNPs) genotypes. Blood samples were collected on FTA cards (Sigma-Aldrich, St. Louis, MO, United States) at three weeks of age from the chicks and genotyped for 600K SNP panel with reference to Galgal 5 reference genome. The FRLC population structure was determined through admixture analysis using the SNP genotypes. Results showed that the FRLC ecotypes are two populations instead of three ecotypes as indicated by the phenotypic morphometric traits. One population is composed of Morogoro-medium and Ching'wekwe (population 1) and the other population is composed of the Kuchi (population 2). Immune response comparision between the populations was done in two appraches; - first, the chickens were infected with LaSota strain of Newcastle disease virus (NDV) at 28 days of age. Tears were collected at 2 and 6dpi days post infection. Blood samples were also collected for serum at 10 dpi. Viral loads at 2 and 6 dpi iwere determined after a quantitative real time polymerase chain reaction (RT PCR) and viral clearance rates were determined. Anti-NDV antibodies levels at 10dpi were determined from the serum samples. Results showed that chickens in population 1 had significantly higher viral loads at 2dpi than at 6dpi compared to chickens in population 2. They also had lower viral clearance rate (VCR) than chickens in population 2. The results further showed that population 2 birds had significantly higher sero-conversion rate than chickens in population 1. There was weak but positive correlation between antibody response and the VCR (0.08) for both population 1 and 2. In the second approach of the experiment, the chicks used in the first part of the experiment were challenged with virulent field strains of NDV on day 34. Viral loads at 2 and 6 days

post infection (dpi), anti-NDV antibody titers, growth rate before and after infection with LaSota strain of NDV and growth rate after infection with virulent field stains of NDV were recorded. Correlation analyses among the traits before challenge with LaSota and after challenge with field strains of NDV were also evaluated. The results showed that population 1 chickens (Kuchi ecotype) had higher mean value measurements for all morphometric traits compared to population 2 chickens (Morogoro-medium and Ching'wekwe) indicating that the linear body measurements can be used for phenotypic selection of the chickens. However, there were individual variations; some individuals had extreme values that overlapped between the chicken populations. The anti-NDV antibody response was also weakly and negatively correlated to lesion scores after exposure of the chickens to virulent strains of NDV. Since results indicated that the chickens are admixed populations with large individual variations, selection for ND resistance chickens is important and requires the use of genetic tools as the canonical selection methods instead of the customary phenotypic methodologies that are being used for selection FRLC in rural areas.

DECLARATION

I, James Richard Mushi, hereby certify to the senate of Sokoine University of Agriculture, that this thesis, which is approximately 14,000 words in length, is my original work and that it has never been submitted nor currently being submitted for degree award in any other institution.

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This declaration is confirmed by

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DEDICATION

This thesis is dedicated my God who gave me health and strength to go through my research work.

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LIST OF ABBREVIATIONS

%	Percent
°C	Degrees Celsius
APMV	Avian Paramyxovirus
DAARS	Department of Animal and Aquacultural Sciences
DNA	Deoxyribonucleic Acid
ELISA	Enzyme Linked Immunosorbent Assay
F	Fusion protein
FAO	Food and Agriculture Organization
FLRC	Free-range Local Chickens
GDP	Gross Domestic Product
GE	Gene End
GS	Gene Start
HI	Hemagglutination Inhibition
HN	Hemagglutinin-Neuraminidase
IBD	Infectious bursal disease
ICPI	Intra-Cerebral Pathogenic Index
ICTV	International Committee on the Taxonomy of Viruses
IFN	Interferon
IgA	Immunoglobulin A
IGS	Intergenic sequences
ISRRP	Iowa Salmonella response resource population
kDa	Kilo Dalton

Kg	Kilogram
L	Polymerase/replicase
M	Matrix protein
MDT	Mean Death Time
mRNA	Messenger Ribonucleic Acid
N	Nucleoprotein
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NO	Nitric oxide
NOD	Nucleotide-binding oligomerization domain proteins
OIE	Office International des Epizooties
ORF	Open Reading Frame
P	Phosphoprotein
PAMPs	Pathogen-Associated Molecular Patterns
PRR	Pattern Recognition Receptors
qRT PCR	Quantitative Real-Time Polymerase Chain Reaction
RIG	Retinoic acids inducible gene1
RNA	Ribonucleic Acid
ss-RNA	Single Stranded Ribonucleic Acid
SUA	Sokoine University of Agriculture
TLRs	Toll-like receptors
UK	United Kingdom
USA	United States of America
UTR	Untranslated Region

vNDV	Velogenic Newcastle Disease Virus
vRNA	Viral RNA
vvNDV	Velogenic Viscerotropic Newcastle Disease Virus
TMB	3,3’, 5,5’-Tetramethylbenzidine
µl	Microlitre

CHAPTER ONE

1.0 GENERAL INTRODUCTION

1.1 Aviculture

Poultry keeping or aviculture is a very important of the agricultural activity all over the world mainly for providing people with meat and eggs and family income. Other benefits include; feathers used as ornaments and making cloths, games such as cock fights, used for rituals by some societies, offered as gifts during ceremonies and they provide manure to fertilize the soil. Among other agricultural activities, aviculture provides about 70% of employment in the rural areas in developing countries (World Bank, 2013).

1.2 Poultry rearing systems

There are three main poultry husbandry systems as shown in Figures 1 a, b, c and d. Intensive system or indoor system is one whereby poultry birds are kept in house and fed for their entire lives. This system is common in commercial production systems and ensures minimal loss of birds by providing all necessary growth requirements such as food, water, medicines and vaccinations. Depending on the weather, controlled conditions are provided to maximize productivity. This is common for layers and broiler birds where pure lines of chickens are kept where flock sizes are often large but not common in FRLC. About 74% of the world broiler meat and 68% of eggs world eggs come from the intensive system (World Watch, 2006). The second system is the semi-intensive system where poultry spend some times outside the chicken house and given supplemental feeds and water as well as treatment and

vaccinations. The third poultry farming system is the extensive management systems where poultry birds roam freely to scavenge for feeds and water in communal lands. The birds are sheltered at night in simple chicken houses or perch in trees around homesteads. They are get very little supplementation whenever present, from kitchen left overs and cereals considered not good for human consumption. Frequently there are no treatments provided against disease or parasitic and worm infestations. On few occasions there can be vaccinations especially against ND. Birds are frequently exposed to dangers of predations, getting lost, stolen, snake bites and accidents. Flock sizes are always small ranging from 2 to 50 birds, thus, a low input system (Yongolo *et al.*, 1996; Aklilu, 2007). The system is predominant in traditional systems practiced more by rural communities (Marwa *et al.*, 2018). This is also the only affordable system available to poor people (Kusina and Mlhanga, 2000).

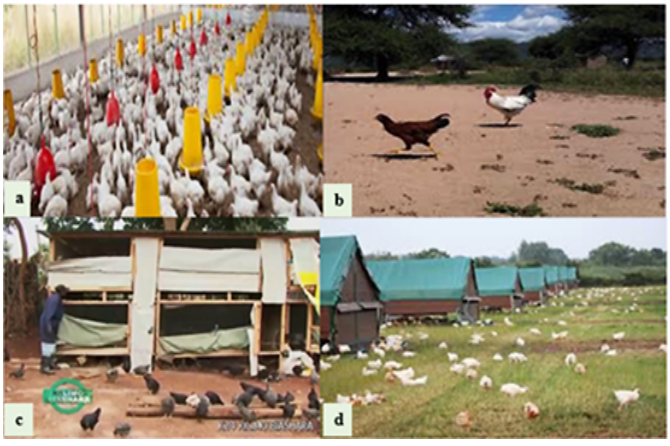

Figure 1.1: a, b, c and d Different poultry husbandry systems in practice, (a) intensive, (b) free-ranging extensive system, and (c) and (d) semi intensive system in local and commercial chickens. ("Compassion in World Farming – Poultry". Ciwf.org.uk. Accessed, October 3rd, 2019)

1.3 Chicken production in Tanzania

According to the Tanzania National Bureau of Statistics (2013), the country depends 30% on agriculture as one of its economic backbone and contributes about 30% of the country's gross domestic product (GDP). One of the agricultural activities is livestock keeping where, over 2,329,942 households keep livestock (National Bureau of Statistics, 2013). According to the bureau, the national livestock census of 2007/2008 for Tanzania mainland, the major livestock species included; cattle, goat, sheep, pigs and poultry. In terms of numbers, there were 21,280,875 heads of cattle, 15,154,120 goats, 5,715,550 and about 1,584,000 pigs. The census indicated further that there were more chickens in the country compared to the numbers of all the large domestic livestock species combined, estimated to be over 72 million chickens (FAO, 2008), making them proportionally the most commonly kept livestock species in the country as shown in Figures 2 a, b and c. The sector has been growing, where, between 1995 and 2003, the growth was 4.3 % per year (FAO, 2008).

There are mainly two types of chickens kept in Tanzania; the exotic breeds/commercial breeds raised in urban and peri-urban areas and the free-range local chickens (FRLC) mostly kept in rural and peri-urban areas (TLMP, 2018; Da Silva *et al.*, 2017).

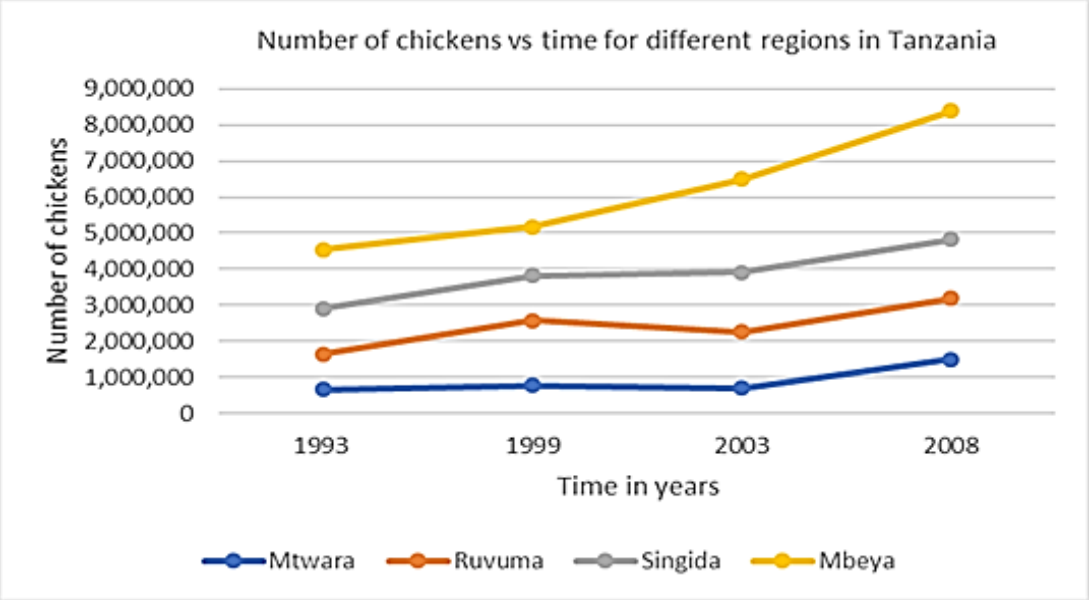

Figure 1.2: Number of chickens kept in four regions of Tanzania mainland in the period between 1993 and 2008 (MOA, 2013)

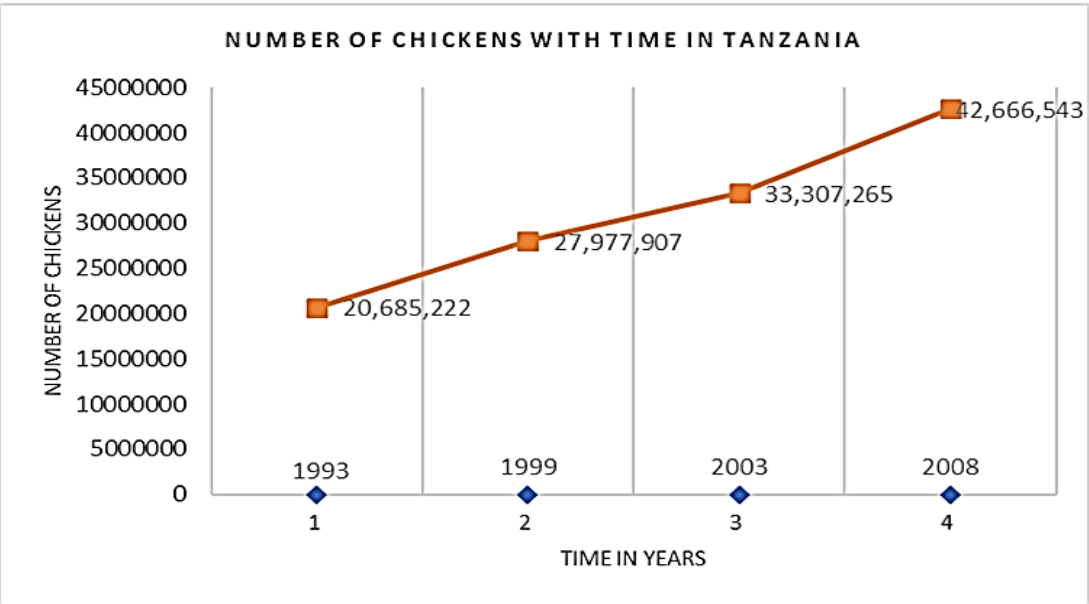

Figure 1.3: Average number of chickens kept in the whole country over a period of 18 years in the period between 1993 and 2008 (MOA, 2013)

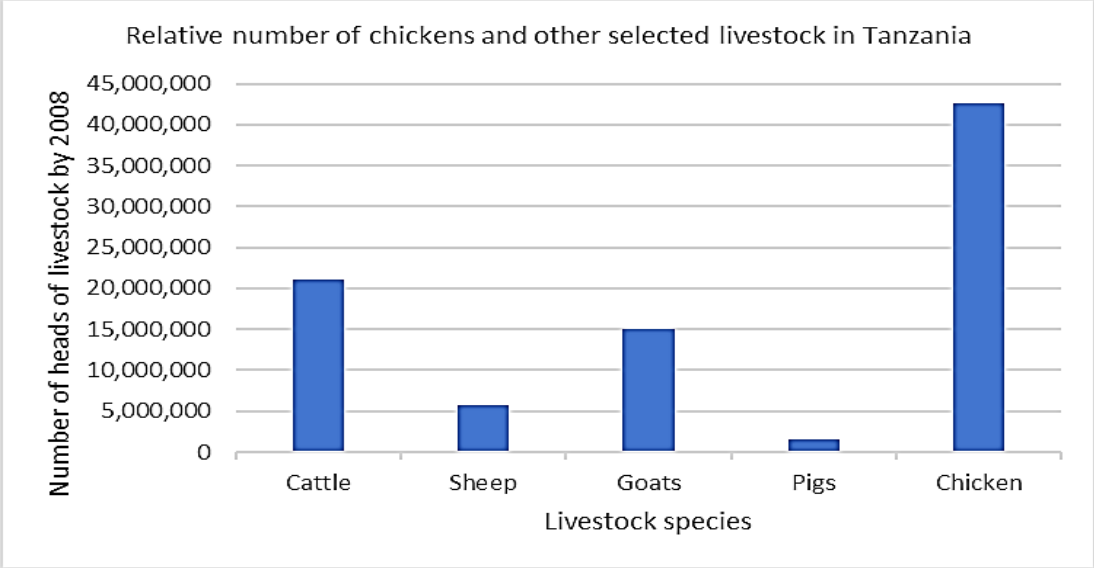

Figure 1.4: Graphical presentations of relative numbers of livestock kept in Tanzania in the period between 1993 and 2008 (MOA, 2013) with chickens occupying the largest proportion.

1.4 Free-range local chickens (FRLC)

Currently, Tanzania has an estimate of over 79 million chickens where about 40 million (51%) of them are FRLC (Ringo and Mwenda, 2018) while the rest are commercial chickens. The FRLC are kept by over 62% of the smallholder farmers and with typical flock sizes of 5-20 birds per household and of mixed ages (Yongolo *et al.*, 1996; Aklilu, 2007). These chickens are locally adapted to certain ecological environments hence referred as ecotypes rather than breeds or lines. The chickens are left to roam around to scavenge for their feeds and water in extensive management husbandry system. There are very little or no established purposive breeding programs for any trait. Selection is based on natural pressures of climatic stress, diseases, and nutrition (Minga *et al.*, 1989). Inspite of their poor genetic improvement, they provide over 90% of poultry meat and eggs in rural African communities (Cumming, 1992; Sonaiya, 1997; Alabi *et al.*, 2006).

The FRLC are diverse in colors, sizes, productivity, and resistance/adaptability to diseases, tolerance to harsh conditions and have aggressive characteristics that enable them to range freely in the environment (Msoffe *et al.,* 2005). As a r*e*sult, there are no pure lines or breeds and thus the names of the chickens in Africa vary from country to country or place to place and even between communities. Tadelle *et al.* (2003) identified five ecotypes in Ethiopia namely; Tilili, Horro, Chefe, Jarso and Tepi named based on their ecological distribution in Ethiopia. Das *et al.* (2003) in Tanzania identified 17 ecotypes through questionnaires where chicken names were based on farmers' experience such as behavior, appearance and even area or locality. Msoffe *et al.* (1998 and 2001) identified five local ecotypes in Tanzania using an approach of observed phenotypic characteristics. He identified; Mbeya, Kuchi, Singamagazi, Morogoro-medium and Ching'wekwe ecotypes. There are different general terms therefore given to the chickens by scientists and publishers and they include; free-range local chickens (FRLC) (Msoffe *et al.*, 2002), indigenous chickens, backyard chickens, rural chickens, village chickens, family chickens, traditional and small holder poultry, scavenging, field chickens and probably more other names (Tadelle and Ogle, 1996a; Kitalyi, 1996; Gueye, 2000; Msoffe *et al.*, 2004; Gausi *et al.,* 2004; Mtileni *et al.,* 2011).

The FRLC sector is a low-input-low-ouput system of poultry farming requiring minimal resources to establish (Branckaert, 2007), thus, they can be kept even by poor households and by people who do not possess land since the chickens move freely in the communal lands scavenging for food and water (Branckaert *et al.,* 2000;

Gueye, 2000; Permin *et al.,* 2001). Despite the wide diversity of rural chickens in the African tropical conditions, the husbandry systems are similar and are mainly extensive and scavenging which accounts for over 80% (Gueye, 1998; Dwinger *et al.*, 2003). The extensive management system being practiced, allows for random mating (Horst, 1990; Williams, 1990) and thus resulting to admixtures of different genetic populations. Frequently, the strongest roosters sire the most offsprings in a population (Williams, 1990).

1.5 FRLCs extensive management system in Tanzania

FRLCs are mainly raised in the extensive management system where the chickens scavenge for feeds and water around in communal lands. Farmers keeping FRLCs engage in mixed agricultural activities whereby they also grow crops and raise other animals. In this production system, farmers are in a disadvantage in getting better market and prices for their chickens and chicken products (Mlozi *et al.*, 2003).

1.6 Diversity among FRLCs

Free-range local chickens in Africa make up to 80% of all chickens kept in the continent (Gueye, 1998; Simainga *et al.*, 2011). They are a huge genetic reserve for future chicken breeding programs for desired traits due to the availability of all chicken genes in these unselected chicken ecotypes (Olori, 2009). The FRLCs are genetically closer to the red jungle fowl (*Gallus gallus domesticus*) than the purposively selected commercial chickens (Minga *et al.*, 1989). In Tanzania there are about 17 ecotypes of FRLCs (Das *et al.*, 2007) which are phenotypically variable with most feature observable in mature birds than the young birds. These

morphological trait differences include among them; growth rate, plumage color and type, body shape and size and egg weights. Some of the characterized FRLC of Tanzania include; Mbeya, Morogoro-medium, Ching'wekwe, Kuchi, Singamagazi, N'zenzegere, Pemba, and Unguja (Kabatange and Katule, 1989; Mwalusanya *et al.*, 2001; Minga *et al.,* 2004; Msoffe *et al.,* 2004).

1.7 Challenges of FRLCs production in Tanzania

FRLCs in Tanzania are raised in extensive management system which exposes the chickens to dangerous conditions that lead to losses of over 50% especially to chicks up to two months of age (Msoffe, 2003; Alfred *et al.,* 2012). At maturity about 80% of a starting flock of chicks will have died (Minga *et al.,* 1989; Rodriguez *et al.*, 1997). Despite being tolerant to many stressful conditions, a major constraint in the FRLC is still diseases (Minga *et al.,* 1989; Sonaiya, 1990b), Newcastle disease (ND) being the number one killer disease (Minga *et al.,* 1989; Musharaf, 1990). Other known diseases include; Infectious bursal disease (IBD) also known as Gumboro disease, fowl pox, fowl typhoid, coccidiosis and helminths (Sonaiya, 1990; Yongolo *et al.,* 1996; Permin *et al.,* 1997). Seroprevalence studies by Minga *et al.* (1989) have shown that of the diseases, ND and fowl typhoid were the most prevalent.

1.8 Newcastle disease (ND)

Newcastle disease (ND) is a highly infectious viral disease of avian species caused by virulent strains of genus Avian orthoavulavirus 1 (AOAV-1) (formerly designated as Avian avulavirus 1 (AAvV-1)) and commonly known as Avian paramyxoviruses 1 (APMV-1) (OIE, 2012; Dimitrov *et al.,* 2019). The disease is endemic in many

countries around the world and dominant in rural poultry production in developing countries than in developed countries (Spradbrow, 1990). It is a mild zoonotic disease with minor symptoms in humans such as mild flu like signs and conjuctivitis (Nelson *et al.,* 1952; Alexander, 2000) that resolves fast even without medication. Chickens show the most severe clinical and pathological signs compared to other avian species (Fuller *et al.,* 2009). The disease is listed in the List A diseases of the Office International des Epizooties (OIE) and requires immediate reporting whenever there is an outbreak (OIE, 2019). It is therefore a major challenge in poultry production systems especially in FRLC. Outbreaks in pigeons and wild birds due to the circulating ppMV-1 which has a potential to transmit the virus into the poultry birds (Brown and Bevins, 2017). Thus, for the disease, it is important to isolate and characterize the virus strains in an area to qualify the area as free from the disease. The Newcastle disease viruses (NDVs) are categorized into five pathotypes depending on the severity of the disease that they cause, they include; viscerotropic velogenic, neurotropic velogenic, mesogenic, lentogenic and asymptomatic enteric strains (Alexander and Senne, 2009).

The viscerotropic velogenic form of the disease is highly pathogenic and is associated with severe gastrointestinal clinical such as diarrhea, ruffled feathers, listlessness, gasping , nasal discharge, swollen face, respiratory rales, and prostrations before death similar to reports by Kommers *et al.* (2003); Wakamatsu *et al.* (2006); Susta *et al.* (2011), the neurotropic velogenic form is associated with severe pathological lesions in the nervous system with clinical signs predominantly as torticollis and high mortality and morbidity (Cattolli *et al.,* 2011). The clinical

signs due to the mesogenic pathotypes infection vary from mild respiratory conditions especially in young birds with low morbidity and low mortality (Alexander, 2003; Cattolli *et al.,* 2011). On the other hand, the lentogenic strains are associated with milder respiratory conditions while the asymptomatic forms do not show any clinical disease (Alexander and Senne, 2009).

1.9 Epidemiology of ND in FRLC

Since its first outbreak in Java, Indonesia (Kraneveld, 1926) and in Newcastle uponTyne (Doyle, 1927), Newcastle disease has become a worldwide disease affecting both poultry and wild birds with frequent epizootics in Africa, Asia, central and south America. In Europe, it occurs sporadically (Naveen *et al.,* 2013; Brown and Bevins, 2017). In the North America, outbreaks of exotic NDV (ENDV) have occurred and eradicated from domestic poultry, the first one in 1971-1973 and second one in 2002-2003 (Chakrabati *et al.,* 2007). The US was free from NDV (Brown and Bevins, 2017) until 2018 and 2019 when new outbreaks occurred in backyard flocks in California (USDA, 2018). However, a country can be regarded free from NDV or regain freedom from NDV three months after an outbreak, provided surveillance has been carried out during that period and no case is reported (OIE – Terrestrial Animal Health Code, 2008b; 2009/2010). In developing countries, better control of the disease is possible in commercial intensive poultry farming systems compared to the extensive free-range systems (Roy, 2012).

In the latter, different poultry species use common feeding and drinking grounds and sometimes share the same shelters at night. This predisposes them to risks of

infections and diseases such as ND (Awan *et al.,* 2007). Sero-surveilance and viral isolations have shown that these birds may harbor all strains of NDV including velogenic strains even in isolated villages and flocks and without necessarily showing clinical signs (Awan *et al.,* 2007). In Tanzania, blood samples from 120 unvaccinated FRLCs of four months of age were tested for antibodies by hemaglutination inhibition (HI) test and found to be 13.3% seropositive (Minga *et al.,* 1989). A similar study by Agbede *et al.* (1992) in Cameroon showed that out of 60 FRLCs from each of three regions were seropositive with percentages of 52, 48 and 47 for equatorial, mountainous and savanna regions respectively. In Germany, higher prevalence was observed with backyard flocks where out of 37 tested birds, 32 reacted positive using HI test (Schobries *et al.,* 1989).

Of the rural poultry species which include ducks, guinea fowls, geese, doves, pigeons, turkeys, pheasnts and quails, the FRLCs are the most susceptible with relatively more severe clinical signs than the other species (Awan *et al.,* 1994). Maintenance of the disease in the flocks is partly attributable to management system and the favorable climatic conditions. Yongolo (1996) reported that ND outbreaks are more frequent in the dry months of the year between June and October in Tanzania with sero-prevalence varying from 25% to 81.5% and varied with respect to months and localities. Other previous studies associated outbreaks with the start of the wet season (Jintana, 1987; Thitisak *et al.,* 1988). A serological survey carried out in Nigeria on unvaccinated local chickens showed that they were seropositive for NDV (Anzaku *et al.,* 2017). In Uganda, outbreaks have been observed more frequently during the hot and dry seasons of the year (George, 1991 and 1992). The

ND outbreaks are unpredictable with no specific season in Ghana, however, sero-surveillance studies showed high prevalence (81.8%) in local chickens (Boakye *et al.*, 2016). In Nigeria, a neighbouring country to Ghana, ND outbreaks are correlated to the Hamattan season when the cold Hamattan winds blow from the Sahara Desert to the West coast (Saidu *et al.*, 2006; Musa *et al.*, 2009). The winds cross the West African countries where they carry dust and help in spreading the NDV leading to stress and outbreaks during the period between end of November and to mid March (Saidu *et al.*, 2006; Musa *et al.*, 2009).

1.10 Persistence of NDV in the environment and its transmission

Temperature and humidity are important conditions for survival of NDV in the environment (Bankowski and Reynolds, 1975). Lower temperatures stabilize the viruses in the environment (Belino and Arambulo, 1968; Brown and Bevins, 2017). The viruses are sensitive to UV light, thus exposure of contaminated material in strong sun light destroys the viruses. In damp or humid and warm chicken feathers, manure, and other materials in chicken house, the viruses can survive for several weeks. In frozen condition the virus can survive forever. NDV can be transmitted by numerous species of birds and animals, insects, human beings and even inanimate objects (Rehman *et al.*, 2018).

Transmission can be from place to place across long distances, thus exported or imported between flocks in a farming unit or among birds in a flock. NDV of different pathogenicities have been isolated from over 250 apparently healthy and sick avian species (Kaleta and Beldauf, 1988). In commercial poultry kept in intensive system, fecal contamination of the feed and water can spread the disease

very fast and cause outbreaks due to bird to bird transmission. Unattended infected carcasses and contaminated poultry products are very crucial in the disease spread especially in commercial poultry (Alexander, 1988b).

Other domestic poultry such as ducks, geese, doves, pigeons, turkeys, peacocks, Guinea fowls, and pheasants are highly involved in the transmission of NDV to the chickens (Warner, 1989; Martin, 1992; Brown and Bevins, 2017) through feeds and water contaminations since they show milder clinical signs and mortality than chickens. Domesticated decorative birds such as Psittacines, pigeons and doves are also a very important source of infection and have been found to shed the viruses for several months and sometimes up to one year (Walker *et al.*, 1973; Alexander and Persons, 1986; Abolnik *et al.*, 2004).

Other non-avian species such as dogs, rodents, cats and foxes which feed on carcasses of NDV infected chickens have been observed to shed the viruses in their feces for up to 72 hours following ingestion of dead infected chickens (White and Jordan, 1963). These animals frequently stray around and can move from village to village scavenging for food therefore becoming a transient source of transmission by contaminating the chicken environments. Various NDV strains have been isolated from pigs in China in the period between 1999-2006 where some were characterized phylogenetically and genotypically (Ding *et al.,* 2010; Yuan *et al.,* 2012). Analyses of the cleavage sequences indicated that the viruses were non-virulent belonging to the vaccine strains (La sota® and V4) commonly used in China. An experiment was set where viral isolates from the pigs were used to infect chickens and were observed to be infective (Ding *et al.,* 2010). It was noted that frequently, chickens and pigs

interact leading to the possibility of cross infections; thus, pigs could also be a potential reservoir of the viruses (Ding *et al.,* 2010). Humans can also get infected although without serious clinical signs but can shed the viruses via ocular secretions (Beard and Hanson, 1984). Virulent NDV strains have also been isolated from insects such as domestic flies which include *Musca domestica* and *Fannia canicularis* collected from within vicinity of infected chicken flock in a backyard (Chakrabarti *et al.,* 2007/2008; Barin *et al.,* 2010). Viruses were isolated and characterized and were found to be virulent strains same as isolates from the infected flock. The viruses have been in the flies' guts and on the surfaces suggesting that flies could transmit the virus mechanically. Flies pick the viruses from birds' droppings and litter material from stables where the chickens visit for egg laying and feeding and also from poorly disposed chicken carcasses.

Inanimate objects that carry the virus for a short period of time such as clothes, boots, wheelbarrows/trolleys, buckets, cars and tracks can carry the virus temporarily and transmit it. Warner *et al.* (1989) reported that the viruses can survive for over eight weeks in hot dry tropical areas with temperatures approching 40°C, and about three months or longer in lower temperatures between 20 and 30°C (Lancaster, 1966). Thus, piles of litter material are a very good reservoir for future infections if disposed untreated (Kinde *et al.,* 2004). Eggs laid by infected birds can also be a source of infection if they are carried from an infected flock to uninfected flock (Meriana *et al.,* 2016).

1.11 NDV heterogeneity

NDV strains characteristically are different virulence, heat stability, shedding ability by host, replication rates, pathogenicity, among others, thus making subpopulations of the viruses (Hanson, 1988). These differences are not transient and the subpopulations are stable in their properties for several generations upon passage, a property that allows the strain to adapt to environmental variations (Martin, 1992). New NDV genetic variants have been constantly emerging due to the broad circulation of the viruses in chicken populations (Dimitrov *et al.,* 2019).

1.12 Control of ND in FRLC flocks

Vaccination is the most pragmatic method for controlling ND outbreaks is FRLC (Munir *et al.,* 2012b). Vaccinated flocks experience less morbidities and mortalities compared to unvaccinated flocks. It is important to vaccinate young birds immediately after the maternal antibodies wane to non-protective levels, and schedules for vaccination be adhered to, from hatching to mature age (Cho *et al.*, 2008). Vaccination protects the chickens from virulent strains during disease outbreaks; however, the chickens become shedders of the vaccine viruses, which become a potential source of infection for naïve birds (Chukwudi *et al.,* 2012). Due to these, some developed countries have prohibited the use of vaccination as prophylactic measure in order to reduce contamination of the environment (OIE, 2012).

1.13 Vaccination challenges in FRLC

FRLC are raised in small flock sizes with multi-ages and scattered around vast areas in villages (Orajaka *et al.*, 1999; Usman, 2002). They are also free-ranging while others have no shelters at all thus making it difficult to catch and vaccinate compared to chickens in commercial settings. Another challenge is the lack of cold chain of handling vaccines in rural areas. Heat stable vaccines such I-2 vaccine or NDV4-HR are more suitable under village conditions where there is lack of cold chain for handling heat labile vaccines like LaSota (Nasser *et al.,* 2000; Alders and Spradbrow, 2001; Illango *et al.,* 2005; Harrison and Alders, 2010; Shim *et al.,* 2011; Xiao *et al.,* 2013).

Cost is another major constraint in FRLC vaccination success. FRLC farmers find the vaccines to be expensive thus they do not vaccinate the chickens, leaving them as reservoirs for infection to uninfected flocks. It is also important to locally produce vaccines in a country, since it is easier to tress their particulars such as dates of manufacture, expiry, storage, administration to chickens as compared to imported vaccines (Alders *et al.*, 2000; Buza and Mwamuhehe, 2001; Dias *et al.*, 2001).

1.14 Natural disease resistance in chickens

Disease control in poultry as in many other animal diseases has been centered on either eradication where flocks are destroyed, vaccination and on drug treatments (Crittenden, 1983; Alders and Spradbrow, 2001; Shim *et al.,* 2011; Xiao *et al.,* 2013). However, diseases are still a problem in FRLCs production systems causing high mortalities and morbidity, high economic losses (Alexander *et al.*, 2012; Chuma *et*

al., 2019; Dimtrov *et al.,* 2019). Farmers and breeders have tried to improve the situation by selecting for resistance to certain diseases under field conditions. Knowledge of genes, gene control of various stress factors in immunity is an important tool to help in selection of chickens using molecular techniques. Therefore, the advent of new cutting edge technologies in genomics and molecular biology has enabled the understanding of diseases and disease mechanisms and selections of animals based on desired traits such as disease resistance.

1.15 Breeding of local chickens for ND resistance

Due to the challenges addressed in vaccination of FRLC, breeding for ND resistant chickens would be a better solution in controlling of the disease. Serological experiments have shown the possibility of presence of ND resistant ecotypes among FRLC (Albiston and Gorrie, 1942; Adeyamo *et al.,* 2012). Hassan *et al.* (2004) reported on the resistance to ND by different breeds of chicken, Msoffe *et al.* (2006) tested five local chicken ecotypes for resistance to fowl typhoid and among them, the Kuchi ecotype showed a better immune response towards the disease compared to the others. Also, Okoye *et al.* (1999) showed that the Fayoumi chickens of Egypt were better resistant to Gumboro as compared to exotic layer type of chickens. This may be an attribute of the host innate immunity to the virus infection designed to retard the viral entry, growth and aid the host in developing specific protection by the adaptive immune responses. Other reports on ND resistance include; Shi *et al.* (2011) who reported the resistance of ND in ducks, and Tsai *et al.* (1992), the genetic resistance to the diseases in turkeys. Natural resistances to other infectious diseases have been reported (Lamont *et al.,* 2002; Minga *et al.,* 2004; Fayeye *et al.,* 2011)

Due to the diversity of FRLC ecotypes found in Tanzania, there are also high possibilities of having better resistant FRLC ecotypes to ND. The diversity observable in the FRLC has also been connected to survivability of some of the chicken ecotypes than others, although, even within an ecotype, some individuals seem more resistant than others, thus, maintaining perpetual generations (Msoffe *et al.*, 2001). A scientific proof is therefore needed and is important to assist in selective breeding for ND resistance in Tanzanian FRLC. If combined with vaccination, productivity in FRLC will be improved and improve rural livelihoods.

However, there are challenges in selection of chickens towards certain desired traits, in a limited study; selection for ND resistance was shown to be negatively correlated to production traits such as growth rate (Lweramira *et al.*, 2009). Some measurable parameters such as the antibodies levels, viral loads and viral clearance rates are indicative of the strength of innate protection in case of viral infection such as NDV. Measurement of the anti-NDV antibody levels, viral loads and viral clearance rates (VCl) among chicken ecotypes following an infection with vaccinogenic strains of NDV are important indicators of immune response during disease outbreaks (Calder, 2007; Salaeo *et al.*, 2018; Salaeo *et al.*, 2019). Viruses that cross the innate barriers stimulate the adaptive immune system which intern express antibodies 10 days after the infection. Stronger innate barriers prevent the virus from entry. Viruses that cross the innate barriers reflect the extent of infection and disease development, thus, chickens with stronger innate barriers will have lower viral loads and higher viral clearance. Thus, genetic markers associated with the anti-NDV antibodies, viral

loads and viral clearance rates can be used for selection of chickens for breeding as a tool for enhancement of resistance to ND.

A wholistic approach must be observed during selection for bredding since, according to Kogut (2009), a very strong immune response might not be necessarily an optimal response. An optimum response can only be determined by measuring the response of the immunological molecules to the pathogen and by assessing the age of the chicken. Also, going for a very strong response is costly in terms of bodily energy that could be directed to other production and reproduction activities.

1.16 Problem statement and Justification

Control of ND in FRLC in Tanzania, like in many other African countries, is still a challenge. Since the FRLC are mainly raised in the extensive management system by rural poor communities where the chickens scavenge for feeds and water in communal lands where risks for ND infection are very high. In practice, the control of ND in these FRLC is through vaccination which is also faced with by many challenges including, lack of cold chains to handle the vaccines, uncertainity in availability, cost as the flocks are typically small and luck of technical expertise. Outbreaks are frequent in these flocks and only survivors restart new flocks. Due to this challenge, ND outbreaks are common in FRLC flocks.

Since there is evidence of innate resistance to other diseases in chickens with ecotype variations and even ND by other poultry species (Kapczynski *et al.,* 2013), it is important to investigate the phenomenon and to select among the Tanzanian FRLC ecotypes for ND resistant chickens for breeding. Alternative methods to complement

vaccination in the control of ND are important to improve FRLC productivity hence improve livelihood in rural communities through nutrition and income. Findings from this study will provide essential input to farmers, chicken breeders and scientists in quest to improve productivity of FRLC ecotypes in Tanzania.

1.17 Research questions

i. Are Tanzanian FRLC similar phenotypically and genotypically similar?
ii. Do Tanzanian FRLC become similarly affected, show similar immune and pathological reaction when infected by NDV?
iii. Are phenotypic traits during infection correlated and heritable by offspring in FRLC?
iv. Are there ND innately resistant chickens among Tanzanian FRLC?

1.18 Objectives of this study

1.18.1 Overall objective

The main objective of this study was to evaluate innate resistance to Newcastle disease in selected Free-range Local Chickens of Tanzania for selective breeding and improved productivity of local chickens

1.18.2 Specific Objectives

i. To characterize three selected Tanzanian FRLCs and to determine their genetic population structure.
ii. To evaluate the immune response against NDV infection among three FRLCs ecotypes of Tanzania.

iii. To determine correlations between phenotypic traits during exposure/infection of the FRLCs with lentogenic and virulent NDV strains and heritability of the traits.

iv. Determine/evaluate innate resistance among the FRLCs.

1.19 Ethical considerations

The study was approved by the Director of Research and Postgraduate Studies Committee of Sokoine University of Agriculture (SUA). It also abided to procedures for handling the experimental animals which was approved by the Institutional Animal Care and Use Committee (IACUC) of University of California Davis prior to the start of the experiments, a partner institution that participated in this research.

1.20 Organization of the thesis

This report has been developed in the publishable manuscript format of the Guidelines to higher degrees of SUA. It is organized into 5 chapters. Chapter one covers the introduction part, literature review, problem statement, and the research objectives. Chapter two has is a manuscript with findings for the phenotypic characterization of the three selected Tanzanian FRLC and their genetic structure. Chapter three is a manuscript that contains findings that show the various immune responses among the three selected chicken ecotypes upon challenge of the chickens with LaSota strain of NDV. Chapter 4 is a manuscript on the correlation of phenotypic traits when the chickens were challenged with LaSota vaccine virus and when exposed to wild virulent strains of NDV. Formats and writing styles are conformant to the journal’s requirements.

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CHAPTER TWO

Phenotypic Variability and Population Structure Analysis of Tanzanian Free-Range Local Chickens

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Abstract

Background: Free-range local chickens (FRLC) farming is an important activity in Tanzania; however, they have not been well-characterized. This study aimed to phenotypically characterize three Tanzanian FRLCs and to determine their population structure. A total of 389 mature breeder chickens (324 females and 65 males) from three popular Tanzanian FRLC ecotypes (Kuchi, Morogoro-medium and Ching'wekwe) were used for the phenotypic characterization. Progenies of these chickens were utilized to assess population structure. The ecotypes were collected from four geographical zones across Tanzania: Lake, Central, Northern and Coastal zones. Body weights and linear measurements were obtained from the mature breeders, including body, neck, shanks, wingspan, chest girth, and shank girth. Descriptive statistics were utilized to characterize the chickens. Correlations between the linear measurements and differences among the means of measured linear traits between ecotypes and between sexes were assessed. A total of 1,399 progeny chicks were genotyped using a chicken 600K high density single nucleotide polymorphism (SNP) panel for determination of population structure.

Results: The means for most traits were significantly higher in Kuchi relative to Ching'wekwe and Morogoro-medium. However, shank length and shank girth were similar between Kuchi and Morogoro-medium females. All traits were correlated with the exception of shank girth in Morogoro-medium. Admixture analyses revealed that Morogoro-medium and Ching'wekwe clustered together as one population, separate from Kuchi.

Conclusions: Phenotypic traits could be used to characterize FRLCs, however, there were variations in traits among individuals within ecotypes; therefore, complementary genomic methods should be considered to improve the characterization for selective breeding.

Keywords: Free-range Local Chickens, phenotypic diversity, genetic diversity, population structure

Background

Poultry plays an important role in the livelihoods [1] of communities in Africa. It is among the most prevalent livestock produced in Tanzania, and chickens account for approximately 94% of poultry raised by farmers [2]. Free-range local chickens (FRLCs) have been produced in Tanzania for many years [3]. In rural communities, the production is mainly for subsistence [2, 4]. Commercial poultry production is common in urban areas where farmers typically raise exotic breeds in intensive systems.

The FLRCs are relatively adapted to and resilient to stressful conditions, including harsh weather and disease [5, 6, 7, 8]. They can be produced with minimal resources, such as shelter, feed, and veterinary services. As a result, they serve as important sources of animal protein and household income, especially for resource-poor marginalized rural communities. The FRLCs and their products are also socially and culturally accepted across different Tanzanian communities. Despite their importance, research on improving productivity of the FRLCs is lacking [9]. Tanzania has over 17 ecotypes of indigenous chickens [10, 11]. Most of these ecotypes have not been well-characterized and their production potential is poorly understood.

There are several methods used to characterize animals ranging from linear measurement of morphological traits to the use of molecular techniques [12]. For instance, morphological measurements have been used to characterize and compare

various poultry breeds [13, 14] and microsatellites have been used to determine the origin of African chickens [15, 16, 17]. In addition, single nucleotide polymorphism (SNP) makers have been used to compare the best method for ascertaining diversity among chickens [18], to cluster the genomes of commercial and non-commercial chicken breeds [19] and to investigate the genetic structure of chicken populations [20]. Climatic conditions and other stressors are highly variable across Tanzania. As a result, FRLC populations may develop different adaptation mechanisms leading to spatial differences in population structure. The extensive management system used to rear FRLCs also allows for random mating leading to panmictic populations [21] with no clearly defined chicken types, strains or lines. The aim of this study was to characterize three Tanzanian FRLC ecotypes using linear body measurements and population structure analysis. Information generated through this study will inform on selection programs to improve FRLC production in Tanzania.

Results

Phenotype characterization

The results of the effects of chicken ecotype and sex on the morphometric and body weight measurement traits are presented in Table 2. Both ecotype and sex effects had a significant influence on traits with the exception of shank girth (SG). Interactions between sex and ecotype effects were only significant for the CG, WS and SL. Males were also significantly different from females for the BL, NL, CG, WS and BW measurements. Similarly, there were significant differences between ecotypes for the BL, NL, CG, WS, SL and BW. Least square means (LSmeans) of the body measurements along with their standard errors (±SE) are shown in Table 3. The

Kuchi ecotype had higher mean values for BL, NL, and BW measurements compared to Ching'wekwe and Morogoro-medium ecotypes, and measurements for these traits were significantly higher in male chickens. The LSmeans for the CG, WS and SL were significantly higher in males across all ecotypes. For the Morogoro-medium chickens, the LSmean for the SL was higher in males, but the difference was not statistically significant. Significant differences in LSmeans for the BL, NL and BW were detected across the ecotypes with the highest LSmeans in Kuchi, followed by Morogoro-medium and Ching'wekwe. There were no significant differences in the SG between sexes across the ecotypes. Overall, males had higher mean measurements across the ecotypes. However, there was individual variation within ecotypes for both sexes that were beyond the means of the ecotypes, where measurements overlapped with other ecotypes. These results indicate that Kuchi chickens were heavier and longer/taller than Morogoro-medium and Ching'wekwe chickens were the shortest and lightest. Linear measurements and body weights within each ecotype were positively correlated except for Morogoro-medium where the SG showed no significant correlation with any other traits (Tables 4a, 4b and 4c).

Population structure evaluation

The admixture analysis for the genetic population structure of the selected Tanzanian FRLC using SNP genotypes indicated evidence of admixture among the FRLC ecotypes (Figure 4). From the analysis, the three chickens' ecotypes clustered into two populations instead of distinct three ecotypes. The Ching'wekwe and Morogoro-medium ecotypes had higher average population proportions of population two (0.78 and 0.75 for Ching'wekwe and Morogoro-medium, respectively), compared to Kuchi

that had a higher average proportion (0.67) of population one as shown in Table 5. Admixture population structure results were supported by the multi-dimensional scaling (MDS) plot (Figure 5), with Ching'wekwe and Morogoro-medium clustering more closely together compared to Kuchi.

Discussion

This study was designed to evaluate linear measurements which could be used to phenotypically characterize three selected Tanzanian FRLC ecotypes based on their morphometrics, key criteria that farmers in Tanzania use to select chickens for breeding purposes. Further, it aimed to enhance understanding of the population structure of the selected FRLC ecotypes using single nucleotide polymorphism (SNP) genetic markers. These results provide a deeper insight int population structure of the FRLCs to complement the use of phenotypic selection. Proper characterization of the FRLC ecotypes, which has not been previously performed due to a lack of known parent stock and reliable source of day-old chicks [33], will promote their commercialization while improving their productivity through aiding in genetic selection of higher performing chickens. In this study, measurements of the BL, NL, CG, SL, SG, WS and BW were evaluated and compared among Kuchi, Ching'wekwe and Morogoro-medium Tanzanian FRLC ecotypes. Ecotype and sex had a significant influence on most of the physical measurements of the chickens (Table 2), similar to observations by Alabi et al [12] for indigenous chickens in South Africa. The males in all groups had the highest mean scores for all the measurements compared to the females (Table 3).

In this study, the Kuchi ecotype had relatively higher mean values for most of the measured traits as compared to the Ching'wekwe and Morogoro-medium ecotypes for both sexes. Ching'wekwe had the least mean values for all measurements. The findings in this study are similar to findings by Lweramila et al [4] who compared the performance of Kuchi and Morogoro-medium under pure extensive management systems as well as findings by Magonka et al [34] which revealed that Kuchi had the highest scores for most measurements compared to Horasi, Naked-neck and Frizzled ecotypes. Based on the results and the physical appearance of the chickens, the Ching'wekwe were shorter than Morogoro-medium and Kuchi, probably owing to their proportionately shorter shanks and body parts. Kuchi on the other hand were observed to have a higher upright stature than the other two ecotypes. Apart from the observed mean variations between ecotypes, there were also large variations observed within ecotypes such that there are some individuals within each group that fell into extremes beyond the group means, thus, overlapping with individuals in other groups. The extreme measurements seen with some individual birds might be a result of random mating in the extensive free-ranging system that leads to admixtures of genotypes and that might have produced the intermediary traits observed in these individuals.

Body weights at maturity were also measured among the chicken types to complement the characterization. The results of this study corroborate previous findings by Lweramila at al [4] and Lyimo et al [17] working with chickens in an extensive husbandry system where Kuchi weighed more than the other FRLC types. As expected, the males had higher mean body weights than the females as observed

in many feeding trials [35]. However, some females in the current study had higher weights than males, probably due to changes in body physiology during laying periods whereby there is increase in the uterus size, fat deposition and increased feed intake [36]. Correlation analyses among the measured traits within ecotypes were positive and high for all traits in Kuchi and Morogoro-medium similar to observations by Alabi et al [12]. However, there was no significant correlation between the SG and other measured body traits (Table 4c). Results of the measurable phenotypic features used in this study could place the chickens into three suggestive ecotypes as they are known from their places of origin suggesting that phenotypic measurable features observed in mature FRLC may be used to complement other methods for identification of chicken ecotypes, especially among the three ecotypes used in this study.

The population structure analysis using admixture analysis placed the three selected FRLC ecotypes into strata of two populations instead of three ecotypes as initially perceived from the phenotypic study. A similar study using microsatellite genetic markers from five Tanzania local chicken ecotypes (Unguja, Pemba, Ching'wekwe, Morogoro-medium and Kuchi), [17] revealed similar findings in which Ching'wekwe and Morogoro-medium clustered together as one population while Kuchi stood as a separate population. Ching'wekwe and Morogoro-medium are found in areas with similar climatic conditions with no natural separation between the chicken types (Figure 1). The lack of geographic barriers, the purchase of seeder flocks from region to region and the free-range management system of the FRLC in Tanzania might have increased the chances of interbreeding between these two

ecotypes resulting in one population rather than two. The Kuchi are more adapted to regions of the Lake and Central zones which are cooler and more humid regions than the Morogoro and Tanga regions where the Ching'wekwe and Morogoro-medium ecotypes are adapted. Studies by Oka et al [37] revealed that the Shamo chicken types of the Shikoku islands and Kuchi share the same mitochondrial DNA haplotypes. Also, Komiyama et al [38] reported that the conformation of Kuchi beak is hooked or parrot-like and sharp like the Shamo chickens of Japan. These studies suggest that the Kuchi chickens might have originated from Japan and formed a breeding colony in the Lake and Central zones where they are adapted. Further, research by Lyimo et al [17] into the origins of Tanzanian local chickens using microsatellite markers found that the Kuchi are the least genetically diverse chicken type among five Tanzanian chickens investigated in the study.

Conclusions

With an exception of the SG, the mean linear measurements of traits used in the current study were significantly different between ecotypes. This indicates that phenotypic trait can be used to identify the different chicken ecotypes. The strong correlations among the linear measurements show that selection for one trait means a selection for the other traits, with the exception of SG in the Morogoro-medium ecotype, which was poorly correlated with other traits.

Individual variation in the measurements within ecotypes with overlap of extreme values between ecotypes was observed, making it difficult to predict a chicken's ecotype. As a result, additional information such as history should be used to

complement the phenotypic characterization. However, from the results, it is difficult to use phenotypic measurable features to assign the FRLC to a particular genetic chicken population. Thus, the selection of FRLC for breeding purposes would be more canonical with use of genomic tools compared to the customary phenotypic methodologies in use by the FRLC farmers in the country.

Methods

Procedures for handling the experimental animals were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of California Davis (# 20831).

Study area

Experiments were conducted at Sokoine University of Agriculture (SUA) in Morogoro, Tanzania using facilities of the Department of Animal, Aquaculture and Range Sciences (DAARS). Three Tanzanian FRLC ecotypes (Kuchi, Ching'wekwe and Morogoro-medium) (Figure 1) were randomly sampled from different zones with varying climatic conditions across the Tanzania mainland. The locations and weather conditions of the different regions and zones are shown in Table 1. Ching'wekwe and Morogoro-medium were sampled from regions in close proximity (Morogoro and Tanga regions) to the Coastal and Northern zones; whilst Kuchi were sampled from the Lake and Central zones (Mwanza and Singida regions; Figure 2) [23].

Experimental chickens

A flock of 389 FRLCs (324 females and 65 males) of the three ecotypes were randomly collected from village households in four regions of Tanzania (Tanga, Morogoro, Singida, and Mwanza regions) and were used to establish a breeding parent stock. Identification of chicken ecotypes was performed as previously described by Msoffe et al., and Guni and Katule [10, 11]. Each chicken was marked with a numbered aluminium wing tag. For each chicken ecotype, a male was placed separately in a pen with 6 to 10 females in a deep litter floor pen. The parent flocks were fed on maize-based layer diets with ad libitum access to water. Routine vaccinations against endemic diseases (Newcastle disease and infectious bursal disease) were administered [22, 24]. Worm infestations, ectoparasites and coccidiosis were treated/controlled using anthelmintics (piperazine DiHCl®, Kepro, Holland), pesticides (imidacloprid Confidor®, Bayer, Holland), and coccidiostats (Trisulmycine®, Laprovet, France), respectively.

Progeny generation chickens

Experimental chicken progenies were established using eggs collected from the parent stock for up to 10 consecutive days. The eggs were labelled with numbers corresponding to a sire and temporarily stored at 18°C before incubation at 60% humidity and 37°C. On day 18 post-incubation, the eggs were transferred to a hatcher with special racks with cubical separations corresponding to sire identity to avoid mixing of chick progenies at hatching. Day old chicks were wing-tagged, weighed and transferred to a bio-secure deep litter floored experimental chicken house where they were fed on commercial chick mash and ad-libitum water access.

Treatment for coccidiosis was performed as needed to control outbreaks in the flock. A total of 1,399 chicks (477 Ching'wekwe, 315 Kuchi, and 607 Morogoro-medium) were produced following five rounds of incubation and hatching for use in the population structure analysis of the three FRLC ecotypes.

Phenotypic linear measurements

Linear measurements were obtained from chickens older than six months (mature chickens) and were performed as described by Geuye et al [25]. In brief, the body length (BL), neck length (NL), chest girth (CG), shank length (SL) and shank girth (SG) were measured in centimetres (cm) for each chicken using a tailor's measuring tape (Figure 3) [26]. The body weights (BW) of the chickens were measured in grams (gm) using a 0.01gm sensitive electronic weighing scale. The linear measurements were performed as follows; BL was measured as the distance from tip of the beak through the dorsum of the chicken to the base of the tail, the NL from the base of the head to the shoulder at the clavicle, CG as the circumference of the chest in front of the thighs, SL as the distance from the hock joint to the metatarsal pad and the SC as the circumference of the middle part of the metatarsus.

Population structure analysis

At 21 days of age, blood samples were collected from each chick by pricking the basilic vein. Approximately five drops of blood were dried on FTA cards (Whatman Biosciences, Brentford, UK) labelled with the chicken's wing tag number and stored at room temperature. Section cuts (3 X 3 mm) using a scalpel blade were made in the cards for each chicken. The scalpel blade was decontaminated in between chickens

via flaming. DNA was extracted and precipitated in sodium acetate ethanol using the phenol-chloroform method [27]. A total of 1,399 birds were genotyped using a chicken 600K SNP Panel at GeneSeek, USA, and quality control (QC) was performed using the Axiom™ Analysis Suite Software version 3.1 (Applied Biosystems, Thermo Fisher Scientific Inc., Calsbad, CA, USA) as explained by Walugembe et al [28]. Briefly, *Gallus gallus* genome version 5 (Thermo Fisher Scientific Inc., Calsbad, CA, USA) chicken genome files were used for comparison during the genotyping of the FRLC. During quality control, SNP set with number of clusters ≥ 2, call rates ≥99% and minor allele frequency ≥0.05 were selected for downstream analyses. Other quality control metrics and imputation of missing genotypes are explained further in Walugembe et al [28]. A total of 396,055 SNPs remained for further downstream analyses. Determination of the structure of the populations was performed using the admixture software [29] as explained in Walugembe et al [28] where briefly, SNPs with closest ancestry were determined using varying values of k (sub-populations) ranging from 1 to 4 and the final k value (k=2) was determined based on the lowest cross-validation error. The population structure was also determined using multi-dimensional scaling in plink [30] in two dimensions as shown by Walugembe et al [28]. At the end of the studies, the chickens were humanely euthanized according to published guidelines [31] and the UC Davis IACUC (# 20831) protocol.

Statistical analysis

The linear measurements were compared among the three chicken ecotypes using R - Statistical Software Program version 3.5.1 [32]. Analyses of variances (one – way

ANOVA) of the least square means with associated standard errors (LSmeans±SE) of the measurements were used to assess for differences among the three chicken ecotypes. Differences were considered significant at $p \leq 0.05$ using the Tukey honestly significant difference (Tukey HSD). The linear model to test the effects of the chicken ecotype and sex on the lengths of the measured body parts was as follows:

$Y_{ijk} = \mu + G_i + A_j + (GA)_{ijk} + e_{ijk}$

where:

Y_{ijk} = trait response variable

μ = general population mean for trait response

G_i = effect of the sex on the trait of an ecotype

A_j = effect of the ecotype on the trait

(GA)ijk = effect of interaction between sex of chicken and its ecotype

e_{ij} = effect of random experimental errors on the trait response

Abbreviations

IACUC: Institutional Animal Care and Use Committee

FRLC: Free-range Local Chickens

ND: Newcastle disease

UCDavis: University of California Davis

USAID: United States Agency for International Development

DNA: Deoxyribonucleic Acid

SNP: single nucleotide polymorphism

MDS: multi-dimensional scaling

LSmeans: Least square means

Tukey HSD: Tukey honesty significant difference

ANOVA: Analyses of variance

QC: quality control

IBD: infectious bursal disease

SUA: Sokoine University of Agriculture

DAARS: Department of Animal, Aquaculture and Range Sciences

Declarations

Ethics approval and consent to participate

The study was approved by the research committee of the Sokoine University of Agriculture and the University of California Davis and followed the Institutional Animal Care and Use Committee (IACUC) protocol of University of California Davis (# 20831) to conduct experiments

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this article are available in the USAID Data Development Library (DDL) repository, and can be accessed publicly at the https://www.usaid.gov/development-data-library request.

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

All authors read and approved the manuscript. JM analyzed the linear phenotype measurements and was the primary author of the manuscript, GC and EM were responsible for preparation of the experimental chickens, and data collection, MW conducted the structure analysis from the SNP genotypes and participated in all phases of the paper write up from conceptualization, analysis to the final write up, EA participated in the structure analysis, HZ is the principle investigator for the Genomics to Improve Poultry Innovation Lab, AM is the principle investigator for this study, PM, RM, HZ, JD, SL, TK and RG participated in the conceptualization of the study and the review of the manuscript drafts before final submission to the journal.

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FRLC	Regions	Location (DD)	Altitude (m)	Av. Temp (°C)[1]	Av. Humidity (%)[2]
Ching'wekwe	Morogoro	-5.5°, 34.5°	213	24.6	75%
	Tanga	-5.0667°, 39.1°	22	28.0	76%
Kuchi	Mwanza	-2.85°, 33.083°	1,363	23.3	76%
	Singida	-5.483°, 34.483°	1,508	22.0	74%
Morogoro-medium	Morogoro	-5.5°, 34.5°	213	24.6	75%
	Tanga	-5.0667°, 39.1°	22	28.0	76%

Table 2.1: Regional sources of parent stock FRLC

[1]Average temperature per year, [2]Average humidity per year

Table 2.2: Analysis of variance p-values for measured traits as affected by the ecotype and sex

BL=Body length; NL=Neck length; CG=Chest girth; WS=Wingspan; SL=Shank length; SG=Shank girth; BW=Body weight, ***p<0.001, **p≤0.01, *p≤0.05

Effects	BL	NL	CG	WS	SL	SG	BW
Ecotype	<2e-16***	<2e-16***	<2e-16***	<2e-16***	<2e-16***	0.1266	<2e-16***
Sex	<2e-16***	<2e-16***	<2e-16***	<2e-16***	<2e-16***	0.0577.	<2e-16***
Ecotype: sex	0.526	0.526	0.0106**	0.0194*	0.000000678***	0.7096	0.426

Table 2.3: Least square means (LSmeans±SE) with standard error of measured traits among the FRLC

Trait	Sex	Ecotype		
		Kuchi	Ching'wekwe	Morogoro- medium
BL	M	50.9±0.62[a]	46.1±0.95[b]	48.30±0.41[c, f]
	F	45.2±0.48[a, d]	43.7±0.22[b, e]	46.80±0.72[c]
NL	M	19.4±0.54[a]	17.0±0.54[b]	17.40±0.28[c, f]
	F	18.0±0.29[a, d]	15.8±0.17[b, e]	16.70±0.45[c]
CG	M	35.30±0.59[a]	31.1±0.45[b]	34.0±0.25[c, f]
	F	29.30±0.20[a, d]	29.0±0.17[b, e]	31.86±0.44[c]
WS	M	47.7±0.78[a]	43.1±0.75[b]	42.74±0.55[c]
	F	45.7±0.82[a, d]	40.0±0.47[b, e]	42.60±0.77[c, f]
SL	M	11.4±0.28[a]	10.2±0.25[b]	10.30±0.16[c]
	F	10.5±0.16[a, d]	9.0±0.09[b, e]	9.90±0.19[c]
SG	M	5.1±0.17[a]	4.1±0.12[a]	4.6±0.06[a]
	F	4.7±0.06[a]	3.9±0.04[a]	4.4±0.13[a]
BW	M	2152.4±50.25[a, d]	1687.6±84.02[b]	2090.4±38.55[c, f]
	F	1575.47±91.37[a]	1162.5±30.65[b, e]	1455.7±68.23[c]

Same superscript small letters indicate no significant difference between mean measurements. First superscript small letters compare among ecotype where the second superscript small letter compares between sex. M = males and F = females, BL=Body length; NL=Neck length; CG=Chest girth; WS=Wingspan; SL=Shank length; SG=Shank girth; BW=Body weight

Table 2.4: Correlations among measured traits in Kuchi ecotype at p≤0.05

Measured trait	BL	NL	CG	WS	SL	SG	BW
BL	1						
NL	0.65***	1					
CG	0.62***	0.41***	1				
WS	0.43***	0.39***	0.34***	1			
SL	0.57***	0.45***	0.34***	0.47***	1		
SG	0.71***	0.53***	0.64***	0.36***	0.36***	1	
BW	0.69***	0.42***	0.77***	0.38***	0.45***	0.8***	1
SG	0.78***	0.68***	0.87***	0.75***	0.77***	1	
BW	0.76***	0.62***	0.808***	0.63***	0.67***	0.83***	1

BL=Body length; NL=Neck length; CG=Chest girth; WS=Wingspan; SL=Shank length; SG=Shank girth; BW=Body weight, ***p<0.001, **p≤0.01, *p≤0.05

Table 2.5: Correlations among measured traits in Ching'wekwe ecotype at p≤0.05

BL=Body length; NL=Neck length; CG=Chest girth; WS=Wingspan; SL=Shank length; SG=Shank girth; BW=Body weight, ***p<0.001 = highly significant

Table 2.6: Correlations among measured traits in Morogoro-medium ecotype at p≤0.05.

Measured trait	BL	NL	CG	WS	SL	SG	BW
BL	1						
NL	0.57***	1					
CG	0.75***	0.46***	1				
WS	0.74***	0.55***	0.69***	1			
SL	0.72***	0.59***	0.66***	0.85***	1		
SG	0.09	0.04	0.07	0.1	0.07	1	
BW	0.74***	0.41***	0.91***	0.66***	0.62***	0.11	1

BL=Body length; NL=Neck length; CG=Chest girth; WS=Wingspan; SL=Shank length; SG=Shank girth; BW=Body weight; ***p<0.001 = highly significant

	Proportions (K = 2)	
Chicken ecotype	**Population1**	**Population2**
Ching'wekwe	0.78	0.22
Kuchi	0.33	0.67
Morogoro-medium	0.75	0.25

Table 2.7: Average proportions of admixture per ecotype

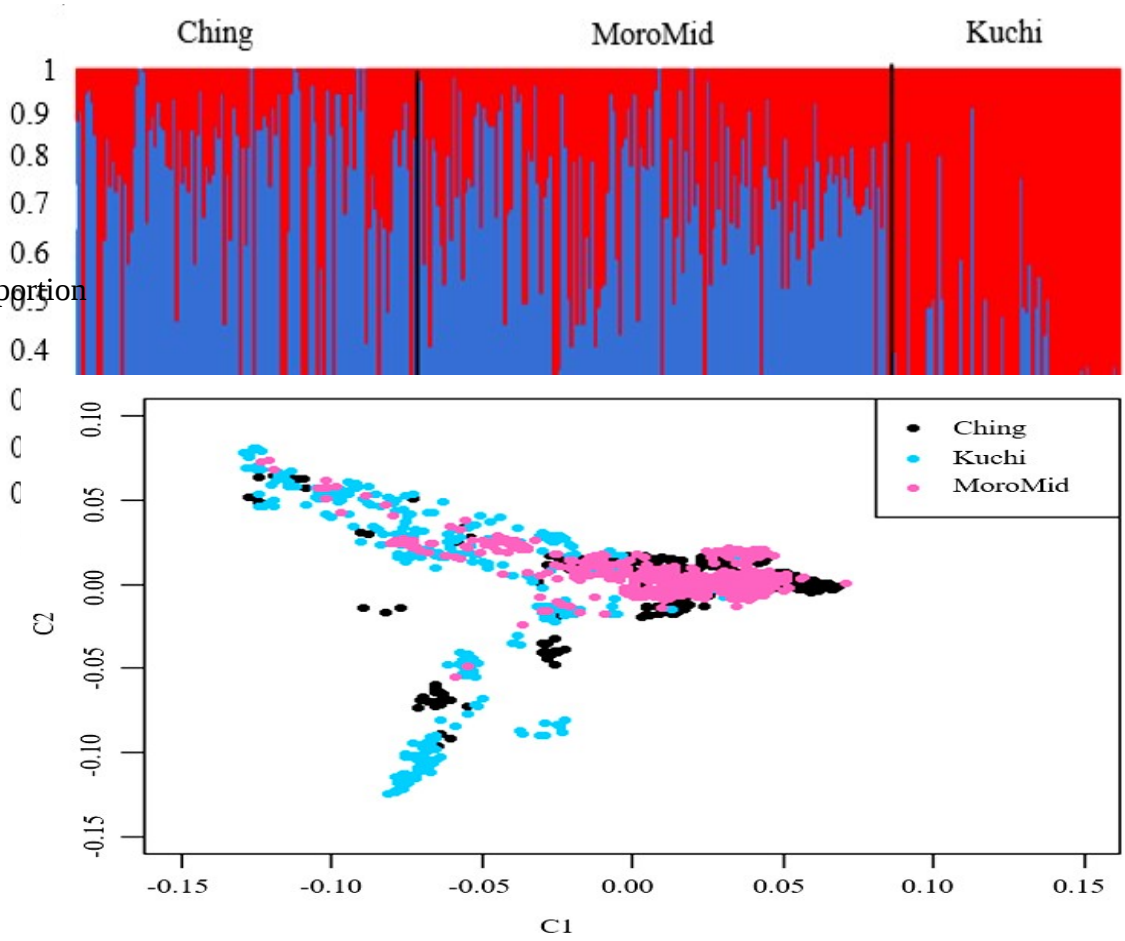

Figure 2.1: Admixture analysis plot showing mixed ancestry among individuals for the three chicken ecotypes; Ching = `Ching'wekwe, MoroMid = Morogoro-medium, Kuchi = Kuchi (Source- Walugembe *et al.*, 2019)

Figure 2.2: Multi-dimensional scaling (MDS) plot showing the distribution of chickens in three clusters of the sampled population. Ching = Ching'wekwe, MoroMid = Morogoro-medium, Kuchi = Kuchi (Source - Walugembe *et al.*, 2019)

Figure 2.3: a, b and c are Ching'wekwe, Morogoro-medium and Kuchi chicken ecotypes respectively

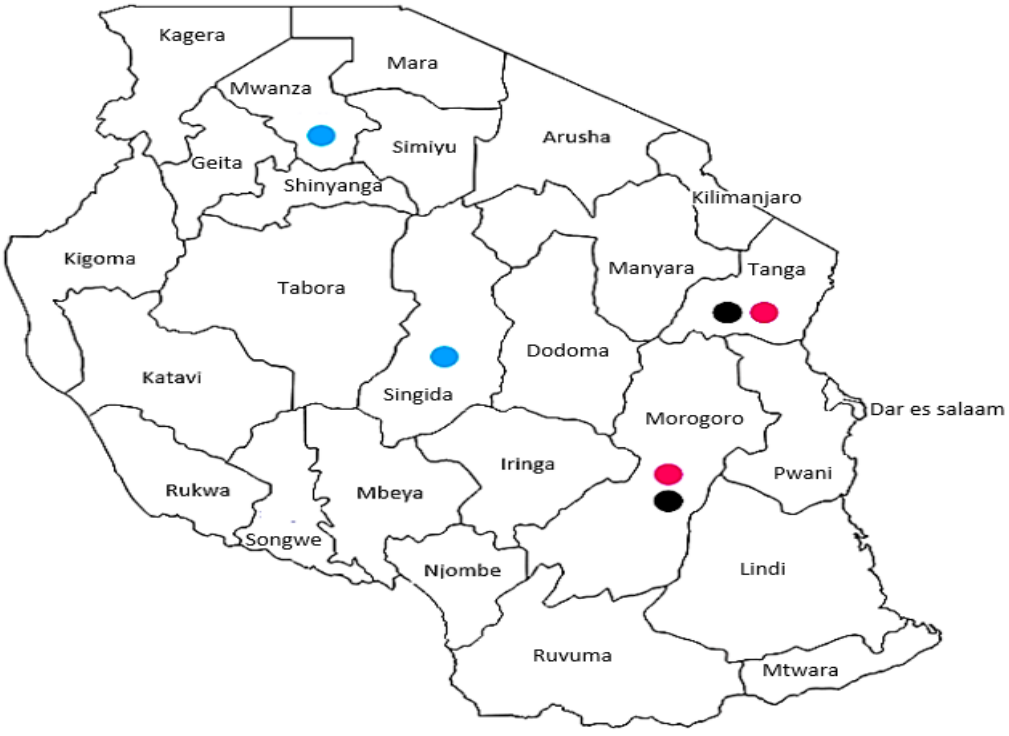

Figure 2.4: Geographical origins of Kuchi *(blue)*, Morogoro-medium *(purple)* and Ching'wekwe *(Black)* chickens in Tanzania (https://d-maps.com/carte. php? num_car=36219&lang =en, 2/3/2020)

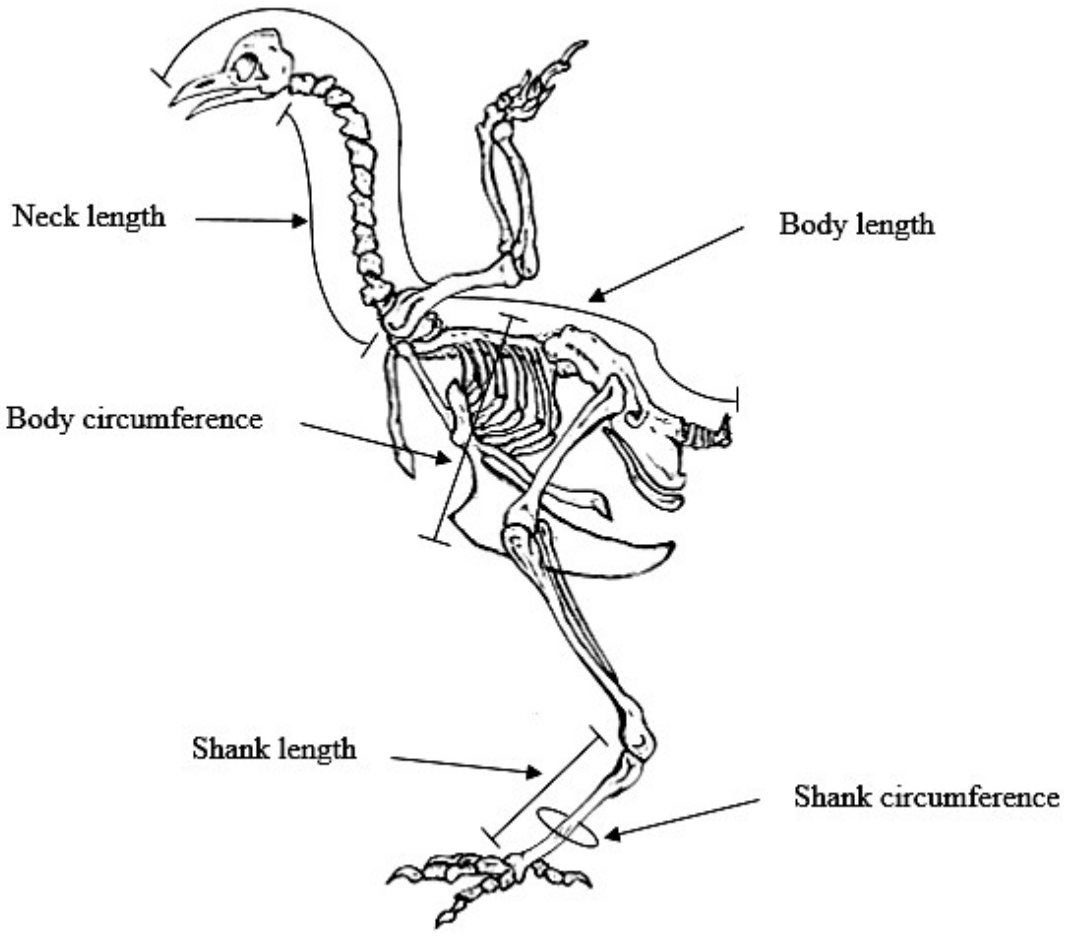

Figure 2.5: Pictorial representation of where various linear body measurements were taken from Tanzanian chickens for their characterization

CHAPTER THREE

Antibody Response, Viral Load, Viral Clearance, And Growth Rate in Tanzanian Free-Range Local Chickens Infected with Lentogenic Newcastle Disease Virus

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Abstract

Newcastle disease (ND) of avian is endemic in Tanzania with frequent outbreaks and losses in free-range local chicken (FRLC) flocks. Selection of breeding chickens with resistance to the disease may improve their productivity especially in rural communities. This study aimed at evaluating antibody responses, viral loads, viral clearance and growth rate of Tanzanian FRLC challenged with LaSota strain of Newcastle disease virus (NDV) as indicator traits for selection of chickens for breeding with enhanced resistance to the disease and economic value. Three popular free-range local chicken ecotypes: Kuchi, Ching'wekwe, and Morogoro-medium from three ecological zones of Tanzania were used for our experiments. Progenies from the chickens were challenged with 10^7 titer of 50 percent egg infectious dose (EID_{50}) of the virus at 28 days of age. The viral loads and viral clearance rates evaluated by qRT-PCR from tear samples collected at 2- and 6-days post infection (dpi) showed that Kuchi could clear NDV better than Morogoro-medium and Ching'wekwe. Anti-NDV antibody levels determined from blood samples collected at 10 dpi using ELISA showed that Kuchi ecotype expressed higher mean anti-NDV antibodies compared to Morogoro-medium and Ching'wekwe. Growth rates determined from body weights collected for 10 days from day of hatch (D0) to 10dpi showed higher growth rate in Kuchi ecotype than for Morogoro-medium and Ching'wekwe chickens. In summary, Kuchi chickens were potentially more resistant to ND compared to Morogoro-medium and Ching'wekwe.

Key words: Free-range local chickens; Newcastle disease; immune response; innate resistance

Introduction

Newcastle disease (ND) is one of the major devastating diseases in poultry worldwide (Ferreira, 2019; Miller and Koch, 2013; Spradbrow, 1993). The disease is endemic in Tanzania and frequently causes outbreaks in free-range local chicken (FRLC) flocks. FRLCs in Tanzania are raised in the extensive management system mainly practiced in rural areas where exposes them to many risk factors like diseases including ND, accidents, mortality at young age, parasitic infestations, predations, poor reproductive performance, and poor growth rates (Sibada and Mapiye, 2005). In addition, extensive management system allows for free movements and interactions of chickens with potential disease reservoirs (Msoffe et al., 2010; Conan et al., 2012). ND together with the other challenges may have contributed to low productivity in Tanzanian FRLCs. Although Tanzanian FRLC have not been extensively studied for improved productivity, they are believed to have high genetic potentials that could be exploited through selective breeding to improve their productivity (Mpenda et al., 2019). The demand for chicken and chicken products in Tanzania as sources animal protein is increasing (TMLF, 2017). Thus, control of ND in Tanzanian FRLC flocks will improve their survivability and productivity, and contribute to availability of animal protein in human diet as well as improved family income. The control of ND in Tanzanian FRLC flocks has been and is still a big challenge due the free-range nature of the husbandry system in practice which exposes them to the risk of ND. Vaccination is the only main reliable approach (FAO, 2001) although vaccination alone cannot fully/effectively control ND in FRLC because of the scavenging system of husbandry. There are also many

challenges to vaccination like poor infrastructure in rural areas, lack of cold chains for storage and transport of the vaccines and insufficient knowledge of vaccinators where, compared to commercial farms where vaccination complemented with biosecurity measures has significantly reduced ND incidences in poultry worldwide (Dortmans et al., 2012, Kiril et al., 2017), it is challenging to control ND in FRLC using the similar approaches. Vaccination in combination with biosecurity measures has been important in control of many diseases including ND and infectious bursal disease (IBD) in commercial poultry (Alexander, 1997) but not practicable in FRLC flocks.

Due to all these challenges, this research focused on evaluating the immune response of Tanzanian FRLC for selection of chickens with improved resistance to ND to complement the vaccination. Previous studies suggest that some FRLC have demonstrated natural resistance to some poultry diseases, including ND (Hassan et al., 2004; Minga et al., 2004; Padhi, 2016). Thus, genetic selection and breeding of Tanzanian FRLC based on disease resistance could offer a complementary approach to vaccination (Zijpp, 1983; Okeno et al., 2012).

This study used phenotypic traits, such as antibody levels, viral loads and viral clearance as some of the key indicators for ND resistance (Pitcovski et al., 2001) which have also been used by other researchers elsewhere. For instance, natural antibody levels have been used as indicator for resistance to avian pathogenic *Escherichia coli* (APEC) in chickens (Berghof et al., 2019), the viral titer at the time of infection and the level of antibodies against a viral agent has impact on the

development of disease (Smyth, 2017). Elsewhere, using similar approaches, some FRLC have demonstrated natural resistance to some poultry diseases, including ND (Hassan et al., 2004; Minga et al., 2004; Padhi, 2016). Modern molecular and serological techniques such as polymerase chain reactions (PCRs) and enzyme linked immunosorbent assays (ELISAs) and also genotyping technologies and genomics have advanced the study of animal genetics and improved animal production, for instance, selection for resistance against diseases in poultry (Jie and Liu, 2011). Using these techniques, Rowland et al. (2018); Salaeo et al. (2018) and Walugembe et al. (2019) have identified many quantitative trait loci (QTLs) that affect response to ND in chickens. With that in focus, this study aimed to evaluate the natural resistance of Tanzanian FRLC to ND using antibody response, viral load and viral clearance rate as indicator traits for resistance to the disease. Three selected Tanzanian FRLC ecotypes challenged with LaSota, a lentogenic strain of NDV used as a vaccine against ND were used in the study. It also aimed at assessing the effect of NDV challenge on growth rate as an important economic parameter in FRLC.

MATERIALS AND METHODS

Animal housing

The experiments were conducted at Sokoine University of Agriculture (SUA) in Morogoro, Tanzania. Experimental animals were kept in the animal facilities of the Department of Animal, Aquaculture and Range Sciences (DAARS).

Experimental chickens and their maintenance

Experimental chickens were selected as previously described by Walugembe et al. (2019). Briefly, a parent stock from three Tanzanian FRLC ecotypes, namely Ching'wekwe, Kuchi, and Morogoro-medium from four regions in four zones across the Tanzania mainland, were used to generate chickens for the experiments. The Kuchi were from Mwanza and Singida regions, Morogoro-medium were from Morogoro and Tanga and the Ching'wekwe were also from Morogoro and Tanga regions representing the Lake, Central, Northern and Coastal zones, respectively. The ecotypes were identified using characteristic features as described by Msoffe et al. (2001; 2004); Guni and Katule (2013). A total of 389 mature chickens (324 females and 65 males) made up the parent stock. All the chickens were uniquely identified using aluminum numbered tags, vaccinated for ND, dewormed and held in collection stations before they were transported to SUA for experiments. Each rooster was kept separately in a labeled pen with 6 to 10 females of corresponding ecotype to make a family and maintained on commercial corn-based layer feeds with *ad-libitum* drinking water. The parent stock generated a total of 1,399 chicks composed of 477 Ching'wekwe, 315 Kuchi, and 607 Morogoro-medium for the experiments.

Preparation of progeny generation chickens

Eggs were collected from the parent flock for periods between 7 to 10 days (less than 10 days old eggs), number-labelled corresponding to the pen numbers of sire to maintain sire identity. For each day, eggs were collected every morning and evening, and subsequently kept at 18°C before being set for incubation. After setting the eggs for incubation, they were candled at day 13 to assess for egg fertility and embryo

viability where defective and non-viable eggs were removed. At day 18 of incubation, the eggs were transferred to racks with cubical separations corresponding to sire identity to maintain chick progenies from mixing at hatching. On hatching, day-old chicks were wing-tagged for identity, weighed and transferred to a biosecured experimental chicken house where they were maintained on *ad-libitum* commercial corn-based chick mash and drinking water. The experiment was conducted in five replicates (rounds of laying and hatching) to obtain large number of experimental chickens (Table 1).

Chickens challenging by LaSota NDV Strain

A viral suspension was prepared from the LaSota strain, a commercial vaccine strain of NDV at a titer of 10^7 of 50 percent embryos infectious dose (EID_{50}) per bird following the methods described by Ramakrishnan (2016) in specific pathogen free eggs. The stock virus was stored at -80°C before the challenge experiments. The chickens were infected at 28 days of age (doa) when the maternal antibodies had waned. A 50µl of the viral suspension were dropped into each eye and nostril to make a total of 200µl for each chicken.

Viral load and clearance assays

Tear samples were collected at 2 and 6-days post infection (dpi) into sterile eppendorf tubes on ice using sterile filtered 200µl pipette tips through irritation of the ocular mucous membranes with crystalline sodium chloride. The samples were stored in -80°C. Ribonucleic acids (RNAs) were extracted from 50µl of tear samples using MagMAX-96™ Viral RNA extraction kit (Thermo Fisher Scientific/Life

Technologies, USA) that uses the magnetic beads technology. RNA extraction was done in RNAse-free environment decontaminated with RNAse Zap® (Ambion®, USA) and quantified by quantitative real time polymerase chain reaction (qRT-PCRs). The RNA quantification were done using the LSI Vet MAX® NDV qRT-PCR Kit (Thermo Fisher Scientific / Life Technologies, USA) with TaqMan™ NDV reagents. The qRT-PCR assays were performed in 7500 fast-real time PCR machine (Applied bio-systems) operated by version 2.3 software. Viral loads were determined using the standard curve method where NDV standards were used to generate a reference standard curve.

Data analyses

Inferential statistics for the chicken populations were determined using the lsmeans package in R studio (R Core Team, 2013, R: A language and environment for statistical computing, R Foundation for statistical computing, Vienna, Austria). The quantification of viral loads at the two time points (2 and 6 dpi), antibody titers at 10 dpi, growth rates before challenge and after challenge, data were expressed as LSmean ± SE. The LSmeans for the titers among the chicken groups were analyzed using one-way analysis of variance (ANOVA) and tested for significances of differences using the Tukey honestly significant difference (Tukey HSD) where *p*-values equal or lesser than 0.05 indicated statistical significant differences among means.

Univariate analyses were performed using linear model to compute the viral loads for the FRLC as least square means (LSmean) with their corresponding standard

errors (±SE) considering the chicken ecotypes, the replicate numbers and their interactions. Viral clearance rates were also determined using the lsmeans package in R as

$$VCL = \frac{VL2 - VL6}{number\ of\ days} \quad [1]$$

Where, VCL is the viral clearance rate, VL2 is viral loads at 2 dpi, and VL6 is viral loads at 6 dpi.

Antibody response measurement

At 10 dpi, blood samples for the determination of anti-NDV antibody response of the chickens were collected as previously described by Walugembe et al., (2019). Blood samples were aseptically collected from the wing veins into sterile eppendorf tubes and decanted overnight for sera collection. Enzyme linked immunosorbent assay (ELISAs) were conducted to determine the titers of anti-NDV antibodies (IDEXX NDV ELISA, IDEXX® Laboratories, Inc., Westbrook, ME, United States). Absorbances were read using a spectrophotometer (iMark TM, Micro-plate Reader, USA). Anti-NDV antibody titers were determined from the sample to positive control ratio (S/P) given by the formula:

$$y = \frac{mean\ of\ optical\ absorbance - Negative\ control\ mean}{Positive\ control - Negative\ control\ mean} \quad [2]$$

Growth rate before and after NDV challenge

Mean growth rate before challenge and after challenge with LaSota NDV strain were determined. For the growth rate before challenge, body weights were collected from

the day of hatch (day 0) to 27 days of age and for growth rate after challenge; the body weights were collected from 28 up to 34 days of age. Individual growth rates (IGRs) were determined according to the formula

$$IGR = \frac{Body\ weight\ at\ day\ t - Body\ weight\ at\ day\ 0}{Number\ of\ days} \quad [3]$$

RESULTS

Viral loads and viral clearance rate

At 2 dpi, the mean log viral load was significantly higher in Kuchi chickens (4.78 ± 0.0571) than in Ching'wekwe and Morogoro-medium (4.67 ± 0.0717). Ching'wekwe had the least mean viral load among the three FRLC ecotypes with mean viral log titer of (4.61 ± 0.0768). There was not significant difference in mean log titer between Morogoro-medium and Ching'wekwe (Figure 1).

At 6 dpi, the mean viral loads were significantly higher for Kuchi chickens (4.05 ± 0.0880) than for Morogoro-medium (4.21 ± 0.094) and Ching'wekwe (4.32 ± 0.070) but not significantly different between Morogoro-medium and Ching'wekwe (Figure 1). Kuchi had significantly higher viral clearance rate (13.3%) compared to Morogoro-medium (9.62%) and Ching'wekwe (8.68%).

Evaluation of anti-NDV antibody levels at 10 dpi

The mean antibody levels were significantly different between chicken ecotypes. Kuchi ecotype had a significantly higher mean anti-NDV antibody level (3.54 ±

0.01) compared to Morogoro-medium (3.50 ± 0.01) and Ching'wekwe (3.39 ± 0.01), respectively (Table 4).

Growth rate among the FRLC ecotypes

The mean (LSmean ±SE) of the growth rate BI (g/day) with NDV was highest for Kuchi (4.30 ± 0.07) followed by Morogoro-medium (4.20 ± 0.06) and least in Ching'wekwe (4.12 ± 0.06). The mean growth rates BI were significantly different between the ecotypes. The mean growth rate AI (g/day) were also significantly different between ecotypes where Kuchi had the highest growth rate AI (6.28 ± 0.21) compared to Morogoro-medium (6.15 ± 0.17) and Ching'wekwe (6.08 ± 0.18) as shown in Table 2 below.

DISCUSSION

This study was designed to characterize three Tanzanian FRLC ecotypes in terms of antibody response, viral load, viral clearance rates and effects of growth rate upon challenge with the LaSota strain of NDV, a non-pathogenic strain of the virus commonly used as a vaccine to protect poultry from ND. The differences observed among the chickens during challenge may be attributable to mean genetic potentials of the different chicken ecotypes to contain infections through innate protection mechanisms which first recognize the pathogen before the adaptive immune system (Chaplin, 2003). Some innate barriers to entry of infectious agents like the NDV viruses and other microbes (Janeway and Medzhitov, 2002) include the physical barriers such as the integrity of the skin, mucus membranes of the mouth, eyes, nose and the mucous itself, anatomical barriers such as the epithelial cell and phagocytic

cells enzymes, phagocytic cells, serum proteins related with inflammation such as the complement system proteins and lectins, cells that release cytokines and inflammatory mediators (Aristizabal and Gonzalez, 2013), among others.

Differences in the abilities of individuals to prevent the infectious agents from crossing the barriers could lead to differences in the magnitude of immune response among individuals and populations (Smyth et al., 2017). Variations in susceptibility to virus infection in this study could have influenced the observed differences in the viral loads at 2 and 6dpi and the viral clearance rate by the chickens This has been observed elsewhere by Filipovic et al. (2017) but using a different disease model. In this study, Kuchi chickens cleared the virus faster than the Morogoro-medium and Ching'wekwe, indicating that Kuchi can probably survive better in case of ND infection compared to the other two. Differences in immune response among local chicken types were demonstrated elsewhere by Hassan et al. (2004) where, using virulent NDV strain and four different Egyptian chicken types. They found that the Mandarah type of chicken had the lowest mortality (20%) compared to the other three, in which the Gimmizah and Dandarawi types had up to 100% mortality. In a different study using infectious bursal disease virus (IBDv) disease model and the same four Egyptian local chicken types, Hassan et al. (2004) also showed that the Mandarah chickens had the lowest mortality rate (10%) compared to the other three chicken types.

In the current study, the high viral titers observed in Kuchi at 2dpi compared to Morogoro-medium and Ching'wekwe may indicate that they can be more susceptible to the infection but they have better resilience than the others as indicated by their

higher viral clearance. However, the viral titers observed in this study in live chickens were high and might be due to the use of less pathogenic strains of NDV and thus might not be seen with more virulent strains.

The mean antibody level, one of the immunological responses in the current study, was relatively higher in Kuchi compared to Morogoro-medium and least for Ching'wekwe. The difference was however significant between Morogoro-medium and Ching'wekwe and between Kuchi and Ching'wekwe but not between Kuchi and Morogoro-medium. The difference in immune response findings observed in this study corroborates findings by Gwakisa et al. (1994) who showed that there were variations in the immune response to NDV infection among Tanzanian ecotypes while different chicken ecotypes were used. In addition, an experiment for assessing the immune response following vaccination against ND showed that local chicken types expressed higher levels of anti-NDV antibodies compared to exotic chicken breeds (Lweramila and Katule, 2004). The variation in antibody response observed in this study could probably indicate that Kuchi and Morogoro-medium immune systems responded better and more efficiently to infection with NDV by expression of significantly higher level of antibodies against the virus as compared to Ching'wekwe ecotype. Considering the significantly high viral clearance rate in Kuchi compared to Morogoro-medium and Ching'wekwe, Kuchi could be more resistant to ND compared to the others.

It is known that the level of neutralizing antibodies detected upon infection by disease agents such as NDV is a good indicator of immunity against that infection (Kapczynski et al., 2013). It has also been shown that high levels of antibodies

against NDV in commercial layer chickens have been shown to protect the flocks against drop in egg production and deterioration of eggshell quality (shell-less, soft-shell and off-colored eggs) (Allan et al., 1978; Stone et al., 1975). The high levels of anti-NDV antibodies observed for Kuchi and Morogoro-medium compared to Ching'wekwe might also indicate/explain their higher relative productivity. The relative differences in immune response among chicken ecotypes in Tanzania have been demonstrated using different disease models and chicken ecotypes. The responses of Kuchi immune system from the current study for instance are in support of observations from a similar study but with a different disease model where Msoffe et al., (2004) showed that among five Tanzanian chicken ecotypes they studied, Kuchi ecotype was relatively more resistant to fowl typhoid following infection with virulent strains of *Salmonella gallinarum* compared to the others. Elsewhere, Okoye et al. (1999) reported that local chickens of Nigeria were more resistant to infectious bursal disease (IBD) compared to exotic chicken types. In the current study, a lentogenic strain of NDV was used, since use of virulent strains might not have yielded results because the chickens would die before sampling and data collection (Msoffe et al., 2004). In a similar study on the immune response against infections in FRLC, Msoffe et al. (2006) found that Morogoro-medium ecotype had higher levels of peripheral leukocytes when infected with *Salmonella gallinarum* compared to other chicken ecotypes they experimented with. We therefore suggest that Kuchi and Morogoro-medium resist NDV infection better than Ching'wekwe ecotype despite the small differences between the ecotypes. Raising these chickens and complementation with vaccination may reduce loses in flocks and improve productivity. Additional research may be required to determine the binding affinity of

the antibodies to their respective NDV antigen to estimate the magnitude of protection. It would also be prudent to perform a similar study with virulent strains of the virus to mimic field conditions. Studies of the immune response to ND in FRLC have some challenges attributable to the non-commercial nature of the local chickens. The lack of appropriate breeding programs leads to massive diversity among the FRLC ecotypes. This could probably be related to previous findings on researches that did not show significant differences in results among the ecotypes as was shown by Mdegela et al. (2002) while working with Salmonellosis in the FRLC.

In this study, it was observed further that the growth rate was not affected by the viral challenge as indicated by growth rate of 2g/day. This growth rate was maintained even after challenge probably because the chickens were resistant to stress and also they were young and still growing. The FRLCs are also known to be relatively more resistant to stress (Minga et al., 2004) than exotic breeds, thus challenging of the chickens with the avirulent strain of NDV may not affect their growth rate. This is contrary to the observations by Liu et al. (2014) and Wang et al. (2015) who reported drop in growth rate when broiler chickens were vaccinated with different doses of NDV vaccine and caused immune stress and weight loss compared to observations in the current study using local chickens. The growth rate observed in this study before the challenge was similar to observation by Magonka et al. (2016) who reported a growth rate of 3.96g/day in Kuchi, whereas in this study, the mean growth of Kuchi was found to be 4.12g/day before infection. The higher growth rate in our study might be attributed to the intensive management system

which the chickens were raised that minimizes energy loss during search of feeds and water compared to semi-intensive system studied by Magonka et al. (2016).

Conclusions

The current study has revealed that Kuchi chicken ecotype is relatively more innately resistant to Newcastle disease compared to Ching'wekwe and Morogoro-medium. The high growth rate in Kuchi offers additional economic selection advantage compared to the other ecotypes. Due to the large variations within and between the chicken groups, selection of chickens for breeding with the aid of genomic tools can identify better chicken genotypes within the ecotypes. An extended work is also needed to unravel molecular mechanisms underlying the virus-host interaction in the different FRLC ecotypes that may assist in selecting the right ecotypes and strains of chickens to be raised in the Tanzanian ND endemic areas. It is also important to explore factors other than the ones accessed in the current study to improve productivity of the chickens in the ND endemic stressful environment.

CONFLICT OF INTERESTS

The authors of this paper declare that they do not have any competing interests.

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	Ecotype		
Replicate number	**Ching'wekwe**	**Kuchi**	**Morogoro-medium**
i	65	57	91
ii	68	67	114
iii	70	77	84
iv	235	102	194
v	124	114	140
Total number of chicks	562	417	623

Table 3.1: Number of chicks produced for the experiments for each FRLC ecotype and for each replicate used for experiments

Table 3.2: Growth rate determined as increase of body weight in grams per days (g/day) compared among the chickens before and after challenge with LaSota strain of NDV.

Ecotype	**N[1]**	**Growth rate BI (LS mean ±SE)[2]**	**Growth rate AI (LS mean ±SE)[3]**
Morogoro-medium	665	4.20(0.060)[a]	6.15(0.170)[d]
Ching'wekwe	623	4.12(0.059)[b]	6.08(0.182)[e]
Kuchi	450	4.30(0.068)[c]	6.28(0.207)[f]

[1]Number of records, [2,3]least square mean ±SE for the growth rate before infection and after infection, Superscript letters indicates levels of significance at $p \leq 0.05$. Along the rows, the growth rate is compared before and after infection within group while along the columns, growth rate is compared between the ecotypes.

Ecotype	**1N**	**LS mean ±SE**
Kuchi	357	0.26(0.013) a
Ching'wekwe	460	0.22(0.013) b
Morogoro-medium	562	0.22(0.014) b

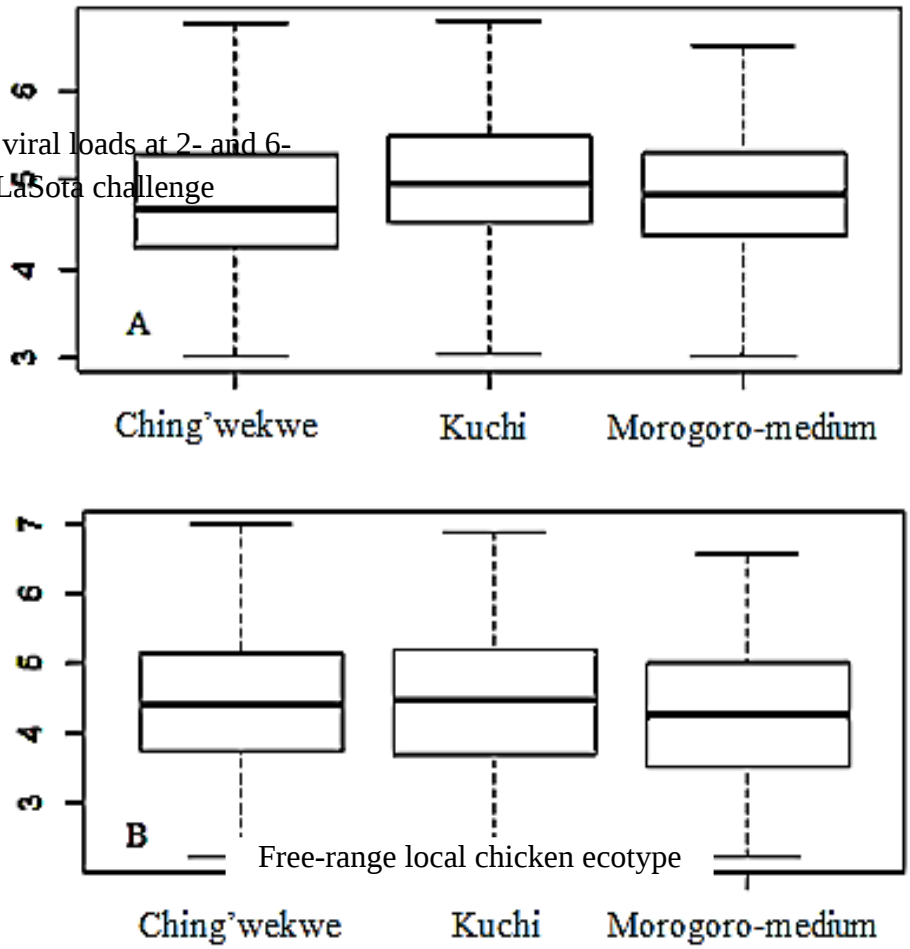
Table 3.3: The viral clearance rate calculated from viral loads at 2 - and 6- days post challenge (dpi) in the three FRLC ecotypes expressed as least square means with associated standard errors (LS mean ±SE).

[1]Number of records, [2]least square mean ±SE for the viral clearance rate at 6 dpi. Compared along the column, the superscript letters indicate the level of significance across the chicken groups at p≤0.05

Ecotype	[1]N	[2]LSmean ±SE
Ching'wekwe	460	3.39(0.01) [a]
Kuchi	357	3.54(0.01) [b]
Morogoro-medium	562	3.50(0.01) [b]

Table 3.4: Mean of log anti-NDV antibody levels expressed as the least square mean with associated standard errors (LS mean ±SE) from samples collected at 10day post infection (dpi) compared between the FRLC ecotypes

[1]Number of records, [2]least square mean ±SE for the antibody titers at 10 dpi. Compared along the column, the superscript letters indicate the level of significance across the chicken groups at p≤0.05



Figure

3.1: a. and b. Comparison of viral loads within the FRLC ecotypes at 2- and 6- days post challenge of the chickens with LaSota strain of NDV.

CHAPTER FOUR

Phenotypic Differences and Relationships Between Responses of Three Tanzanian Free Range Local Chickens to Infection with Lentogenic and Velogenic Strains of Newcastle Disease Virus

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ABSTRACT

Aim: Evaluate relationships among phenotypic traits in Tanzanian free-range local chickens during lentogenic and velogenic Newcastle disease virus infections.

Methods: Three ecotypes of Tanzanian free-range local chickens; Ching'wekwe, Kuchi, and Morogoro-medium were sampled from three eco-climatic zones of Tanzania and used to produce a population of 1,399 chicks composed of 477 Ching'wekwe, 315 Kuchi, and 607 Morogoro-medium. However, from previous population structure analyses studies, Ching'wekwe and Morogoro medium were genetically similar thus placed in one population (population 1) and Kuchi as separate population (population 2). Experiment were composed of two stages; in the first stage of the experiment, chickens were challenged with the LaSota vaccine strain of Newcastle disease virus in four trials. Various phenotypic traits including viral loads at 2- and 6-days post infection, anti-Newcastle disease virus antibody levels at 10dpi, viral clearance rates and growth rates were evaluated. The second stage of the experiment involved exposure of stage one birds to velogenic field strains of Newcastle disease virus. The growth rates, mortalities, postmortem lesion scores and survival rates were measured following velogenic strain exposure. The differences between ecotypes for various Newcastle disease virus response traits and phenotypic relationships among the traits were estimated.

Results: For velogenic NDV exposure, the mean growth rate dropped by 10.2g/day. Mean lesion scores were highest in the intestines (0.81) and lowest in proventriculus (0.10). There were significant differences in lesion scores ($p \leq 0.05$) between population 1 and 2 for average lesion score, post-exposure growth rate, proventriculus, tonsil and intestine. There were no significant differences in mean

lesions for trachea between populations. Populations 2 chickens lost significantly more weight than population 1 on exposure to vNDV. Genetic correlations were not significantly different from zero. Phenotypic correlation was significantly positive and moderate between the pre-challenge growth rate and post-challenge growth rate. The post-challenge growth rate was positively phenotypically correlated to average lesion scores (0.12) and negatively correlated to the viral clearance rate (-0.12) following exposure to vNDV.

Conclusions: The Kuchi chicken ecotype had higher mean responses for the viral clearance rate, and antibody response assessed compared to Ching'wekwe and Morogoro-medium. However, the response to infection with the LaSota strain of Newcastle disease virus was not highly correlated with response to subsequent exposure to velogenic field strains of Newcastle disease virus in the free-range local chicken ecotypes. Significant differences were observed for mean lesion scores after exposures to vNDV between the populations.

Key words: Newcastle disease, host response, free-range local chickens, phenotypic correlations

INTRODUCTION

Newcastle disease (ND) is an avian disease caused by avian paramyxovirus type I (APMV I) which affects over 256 species of birds across the world [1]. Chickens show more severe clinical signs and mortalities compared to other avian species [2]. The disease is ranked as the fourth most important poultry disease in terms of the number of livestock units lost for poultry species, behind the highly pathogenic avian influenza (HPAI), infectious bronchitis (IB), and the lowly pathogenic avian influenza [3]. The severity of ND depends on the virulence of the NDV strain causing the infection [4] thus producing four clinical forms of the disease namely; Doyle's form (or viscerotropic-velogenic), Beach's form (or neurotropic-velogenic), Beaudette's or mesogenic form and Hitchner's or lentogenic form [5].

The disease is a big problem affecting free-range local chickens (FRLC) in Tanzania due to the extensive management system that exposes them to risks of infections. The disease is often controlled through vaccination despite the many challenges of vaccinating FRLC. Chicken vaccination is inadequate and often faced with various challenges, including; high cost of the vaccines since mainly they are manufactured for commercial farming systems hence large doses for large flocks compared to small flocks of different ages in FRLC, lack of cold chains to handle vaccines under village conditions [6] and failure of the current vaccine strains which are several generations older and failure to protect against current circulating strains [7]. The insufficient protection of the FRLCs against diseases such as ND affects their production therefore many farmers keep them mainly for subsistence use. Selective

breeding for ND resistance along with production traits could be used as a tool to improve productivity of FRLC.

Immune response to NDV infection is known to vary with the host type, individual differences, viral dose and strain causing the infection [8]. Host genetic variations in resistance to ND within and between host breeds have been reported by [9]. [10] and [11] found that there were individual differences on genetic resistance to ND within chicken breeds while Hassan et al., [12] and [13] reported on the difference in ND resistance among chicken breeds. Adeyemo et al. [14] using two Nigerian chicken ecotypes, reported that the Yoruba ecotype chicken expressed higher anti-NDV antibodies compared to Fulani ecotype and exotic breeds which they compared with. Using Kuchi, Morogoro-medium and Ching'wekwe embryos, Schilling et al. [15] reported that Ching'wekwe had significantly lower NDV load at after 72 hours compared to Kuchi with Morogoro-medium having the highest load indicating differences in immune response to NDV infection.

Further, in-vitro studies with virulent NDV on chicken cells have shown strong innate immune responses such as expression of nitric oxide (NO) in heterophils [16] and peripheral blood mononuclear cells [17], gamma interferon (IFN-γ) mRNA in peripheral blood mononuclear cells [17], and alpha (IFN-α) and beta (IFN-β) interferon mRNA in macrophages [18] which are important for the protection of chickens during infections.

The strength of the immune response towards infection is important to ensure the survival of chickens after infections. It is known that favorable production traits are

negatively correlated to the antibody response to ND [19]; however, selection for optimal immune response and production is important. The response to ND in Tanzanian FRLC have not been well studied, therefore there is no sufficient information on Tanzanian FRLC that can guide farmers, scientists and the chicken breeders on selection of FRLC for breeding in order to improve their productivity. Due to this information gap for the Tanzanian FRLC, this research aimed at evaluating the phenotypic traits response correlations among three Tanzanian FRLC ecotypes when challenged with LaSota, a vaccine for ND, followed by velogenic field strains of NDV. Results will provide insights into the immune system and growth responses of the FRLC during ND infections to help in the selection of the chickens for their improved productivity.

Materials and Methods

Experimental design

Management and handling of experimental chickens followed procedures approved by the Institutional Animal Care and Use Committee of University of California Davis (#: 17853). Three Tanzanian FRLC ecotypes, including 315 Kuchi, 607 Morogoro-medium and 477 Ching'wekwe were used as described by Walugembe et al. [20]. All the chickens were genotyped as described by Walugembe et al. [20]. Briefly, blood samples were collected from all the chickens on Whatman FTA cards (Sigma-Aldrich, St. Louis, MO, United States) at 21days. Genomic DNA was extracted, genotyping done using the Affymetrix Axiom® 600k Array at GeneSeek (Lincoln, NE, USA) and genotype data quality filtering was performed with Axiom™ Analysis Suite 3.1 (Applied Biosystems, 2018).

Challenge experiments

The experiment was conducted in two stages; the first stage involved challenging of chickens with live attenuated type B1 LaSota lentogenic NDV strain. Immune response traits including viral loads at 2 and 6days post infection (dpi), anti-NDV antibody levels at 10 dpi, viral clearance rates and growth rate as described by [20]. The second stage of the experiment involved exposure of the chickens to virulent field strains of NDV. The chicks from the LaSota trial were kept for at least four months to wane the acquired anti-NDV antibodies to non-protective titres and were exposed to virulent field strains of NDV. The NDV velogenic exposure trials were conducted in three batches at different time points; batch one was a combination of birds from replicates 1, 2 and 3 of the LaSota challenge experiment, batch two consisted replicate 4 chickens while batch three consisted of replicate 5 chickens. The chickens in batch one was seven, six and three months old respectively at the time of exposure to vNDV. For batches two and three, the chickens were three and three and half months old, respectively.

The non-protective acquired anti NDV antibody levels were determined from sera samples collected from 50 randomly selected chickens for every batch of exposure using ELISA technique. IDEXX NDV ELISA (IDEXX Laboratories, Inc., Westbrook, ME, United States). The monitoring of the acquired anti-NDV antibody levels were done every week until the day of exposure to natural infection by vNDV. The variation of time of exposure to the field strains of vNDV among the flock batches after the three months of waning of acquired anti-NDV antibodies depended

on the availability of live infective sick chickens from the markets. For each batch in natural exposure trial, chickens from the three ecotypes were pooled together in a 50 square meter area bio-secured chicken experimental room and raised under similar conditions with access to *ad libitum* clean feeds and water. Chickens were infected with velogenic NDV using infective chickens (seeder chicken flocks) created from ND naïve chickens.

Seeder chicken flock

Sick infected chickens were randomly collected from the market in Morogoro metropolitan and confirmed for presence of ND through amplification of the fusion (F) protein gene with F gene specific primers. Five sick chickens were mixed with 50 NDV naïve chickens for four days until they showed clinical signs of ND to create infective seeder flocks. Infections in seeder chickens were confirmed through clinical signs and PCR amplification of the F-gene.

Natural exposure/infection of experimental chicken flocks

Seeder NDV infected birds were mixed with the experiment chickens at a ratio of 1 to 10 chickens. Monitoring was done for 21 days after exposure and various NDV response traits including clinical signs, period from infection to death and postmortem (PM) lesions were recorded daily in the mornings and evenings. Body weights were recorded on intervals of two days and growth rates were computed from the body weights by a linear regression of the weights on the ages of the chickens. 21 days after exposure of the chickens to natural infection, surviving chickens were euthanized and lesions were scored.

Postmortem examination and lesion scoring

Postmortem examination (PM) lesion scoring system (Table 1) was done as explained by Hussein et al. [21], however slightly different. Hemorrhagic lesions were scored from the cartilage rings of the trachea, mucous membranes of the proventriculus especially at the bases of the papillae of the posterior and anterior orifices, ecchymotic hemorrhages and necrosis of the payer's patches in the small intestine and the cecal tonsils.

Correlations of traits and heritabilities

Phenotypic correlations between the trait responses during LaSota NDV strain infection and natural infection with the virulent field strains of NDV were estimated. In the first step of the statistical analyses, the best models to fit the data were determined. Therefore, since all the chickens in this current study had been genotyped, the population proportions were fitted as fixed covariates in the models, rather than fixed effects of ecotypes. For each trait, a univariate linear model was run that included the fixed effects of the replicate, sex, and assay plate (only applicable for the anti-NDV antibody level and viral load), population admixture proportions fitted as covariates, and random effects of the chicken (genetic) and its dam (maternal effects). Dams were assigned using the procedure explained in Walugembe et al. [20]. The models were given as:

$$y = X_1 u + X_2 b + Z_a a + Z_m m + e$$

where **y** is the vector of phenotypic measurements, $\mathbf{X}_1$ is a vector of ones, $\mathbf{X}_2$ is the incidence matrix relating the fixed effects and covariates to **y**, **b** is the vector of fixed

effects and covariates, **Za** is the incidence matrix relating the phenotypic observations to the vector of random bird effects, **a**, with a genomic relationship matrix to explain the (co)variance among birds, **Zm** is the incidence matrix relating the phenotypic observations to the vector of dam effects, **m**, and **e** is the vector of random residuals. The dams and random residual effects were assumed to be independent. The significance of fixed effects and covariates for each trait was determined using the REML procedure of the ASREML software R package version 4.1 [22]. Effects significant at $p < 0.05$ were kept in the model. Significance of the random effects of dam in the models was determined by comparing likelihoods of full and reduced (excluding the dam) models. Based on this, the final model was selected for each trait and were used to set up all pairwise bivariate animal models, which were used to estimate phenotypic and genetic correlations. All models were implemented in ASREML 4.1 [22]. Heritabilities were also computed.

Survival analyses

Survival days (SD) for each chicken were taken as the number of days from the introduction of seeder chickens into the flock of experimental chickens to the time of death. A Cox proportional hazards regression model was run to determine the significance of the effects of population proportion on survival days.

$$SD_{ij} = \mu + SEX_i + REP_j + \beta \cdot Pop + e_{ij}$$

Where SD is a survival days record, Sex is the sex of the chicken, Rep is the replicate the chicken was from (1 to 5), Pop is the proportion of each chicken's genotype in the population that was from the first ancestral population, as determined

by Admixture analysis, and *e* is the random residual error term, assumed to be distributed as N (0, **I** σ_e^2). This model was implemented with the "Survival" package in R. We also ran the following linear mixed model:

$$SD_{ij} = \mu + SEX_i + REP_j + \beta \cdot Pop + Z \cdot u + e_{ij}$$

Where *u* is the random polygenic effect of the chicken, which was assumed to be distributed as ~ N (0, **G** σ_G^2), and accounted for the (co)variances between animals due to genomic relationships. G is a genomic relationship matrix computed as described by VanRaden method 1 [23], and all other terms are as described for the model above. This model was implemented in ASReml 4 [22].

Results

Descriptive statistics of populations

In batch one of the exposures to natural infection with vNDV, 16% of Morogoro-medium, 25% of Ching'wekwe and 14% of the Kuchi died. For batch two, 32% of Morogoro-medium, 23% of Ching'wekwe and 43% of Kuchi died, while 29%, 15% and 38% for Morogoro-medium, Ching'wekwe and Kuchi, respectively died in for batch three. The estimates of the phenotype traits for the ecotype proportions are shown in Table 2. The mean growth rate (GR) was -10.2 g/day. Individual variations in GR ranged between 21.4 and -85.7 g/day. Average lesion score at postmortem examination (PM) were high in the intestine and the cecal tonsils (0.82 and 0.71 respectively) and low in trachea and proventriculus (0.53 and 0.10 respectively). The overall mean lesion score was 0.54, with scores ranging from 0 and 2.38.

Ecotype proportion, replicate and sex of the chickens, had significant effects on the post-exposure GR, AVL and tracheal lesions. Chickens in population 2 increased post-exposure GR by 8.6 g/day compared to chickens in population 1. Chickens in replicate 4 grew at 12.7g/day compared to chickens in replicate 5. Replicate 4 chickens lost the most weight (-13.6 g/day) followed by those in replicate 1 (-1.6g/day). Average lesion score was affected by sex. The males had higher average lesions score (0.13) compared to the females. Replicate 4 chickens had higher average lesion score than chickens in replicate 5. Chickens in lower mean lesion scores not different from zero. Mean lesion scores in the trachea were lesser than lesion score in chickens of replicate 5. Mean lesion scores in chickens of replicate 1 and 4 were similar to lesion score in replicate 5.

Heritability of traits

Heritability for the phenotypic traits of the chickens during infection with LaSota strains of NDV have been reported by Walugembe et al. [20]. Statistical summary (±SE) and heritability estimates of variance components of traits during natural infection with the field strains of NDV are shown in (Table 2). Heritability of post exposure growth rate was moderate 0.17. Heritabilities of the lesions scores were close to zero and the dam effects were not significant.

Phenotypic traits correlations

Genetic correlations were not included because their heritabilities too were not significantly different from zero. Estimates of phenotypic traits correlations during lentogenic infection and exposure to vNDV (±SE) are presented in Table 3. Average

lesion score was highly correlated to lesion scores in the small the intestines, trachea, proventriculus and cecal tonsils with correlation coefficients of 0.73 for the small intestines, 0.58 for the trachea and 0.52 for both the proventriculus and cecal tonsils, respectively. Pre-infection GR was positively correlated to the post-infection GR (0.47) and anti-NDV antibodies levels at 10dpi (0.12). The mean anti-NDV antibody levels at 10dpi had low negative correlations with growth rate, average lesion score and mean lesion scores in the trachea, proventriculus and intestines.

Survival analyses

Sex of the chickens, replicate of exposure to natural infection and the ecotype proportion have significant effects on the survival of the chickens. The hazard ratios (HR) are shown in Figure 1. Males were 1.4 times more likely to die of ND compared to females. Replicate 3 chickens were 0.24 times more likely to survive than chickens in replicate 1 and 4. Population 2 chickens with a HR of 0.52 had higher chance to survive compared to chickens of population 1. The other replicates were not significantly different from each other.

Discussion

The negative growth rate in all the chicken groups could be attributable to damage of the cells lining the digestive tract. The virus is known to grow mostly in the organs of the digestive and the respiratory systems with higher preference to the digestive tract causing hemorrhages and edema in the proventriculus and small intestine [24], epithelial cells degeneration and crypta, and atrophy of the villi [25] that could lead

to anorexia, lowered digestibility, poor feed conversion potential, diarrhea, weight loss and impaired GR in the infected chickens as observed by Sedeik et al. [26].

Exposure of the chickens to field virulent strains of NDV was highly detrimental to the GR of the chickens where on average, the chickens lost about 10.2g/day. Contrast to the artificial infection experiment, the infectious dose in the natural exposure trial was not predetermined. It could be that viral titers at natural infection were very high than those of the field strains if NDV were too virulent and overwhelmed the chicken immune systems. The Morogoro-medium and Ching'wekwe chickens from population 1 chickens had relatively lower GR compared to the Kuchi chickens of population 2 and were also not differentially affected by the infection stress. This was similarly observed for the antibody titres and viral clearance rates following the challenge with LaSota strain of NDV.

Mortalities in the flocks started at 4 days post exposure (4dpe) as peracute infection which does not show observable clinical signs contrary to findings by Brown et al. [27] and Kommers et al. [28], where they reported presence of severe peri-ocular edema in chickens at 2 days post infection (2dpi) and necro-hemorrhagic plugs in ceca adjacent to the cecal tonsils and multi-focally within the small intestine at 4dpi. In this study, clinical signs and gross lesions were evident from the second day of the onset of mortality contrary to findings by Brown et al. [27]. However, the most obvious hemorrhagic lesions observed in this research were in the Larynx and trachea, the proventriculus, the intestines and the caecal tonsils and were similar to previous finding ([29]; [28]; [30]. The hemorrhagic lesions had different severities

that varied among individual chickens. The variations observed in the chickens could be attributable to other factors such as the virus strains from the infectious sick chickens which were fields strains and that possibly the infections were caused by more than one viral strain with varying pathogenic activities as also reported by Wakamatsu et al. [31]. The variations could also be caused by differences in the innate protection of individual chickens [32] since the FRLC used in the experiments were not pure breeds and were from different ecotypes [20] which could have different innate immunity potentials to fight against the infection and diseases. Infection of chickens with virulent strains of NDV elicits strong innate response against the viruses [33] which in contrary, the chickens in this experiment developed severe pathological lesions. It was expected that all NDVs through expression of gene V can suppress interferon type I which is responsible for regulation of innate immune response to the viral infection [33]. The average lesion scores assessed in the various organs in this study were generally low (0.70) but with great variations among individuals (0 to 2.38). The scores were also lower than mean scores observed by Hussein et al. [21] using a similar scale, however he experimented with broiler chickens which are known to be less resistance to diseases than the FRLC used in this study. Exotic breeds are often pure breeds selected for specific traits especially production. This channels vital resources towards the selected traits and less is given to the immunity traits [34] contrary to the FRLC ecotypes which are not selected for any specific traits. The variation of the lesion scores within the FRLC might be due to individual differences in strength of the innate immune response to the infections which would determine the chicken’s susceptibility the infection and hence the viral load severity of the disease and extent of pathology. Postmortem

lesions depend on the stage of the disease where, at early stages of the disease, only mortalities were observed without any other visible pathological lesions, and the lesions became more obvious at later stages of the disease. The minimum lesion score for the intestine was 1 and the highest was 3, with a similar observation in the cecal tonsils. The proventriculus had lesions scores ranging from 0 to 3 while the trachea had a score between 0.5 and 3.

Correlation studies for various traits in chickens have been studied especially in exotic breeds of chickens [35]; [36]; [37]. In this study, the pre-infection GR was also weak and negatively correlated to the post exposure GR (-0.10) further, the correlation between the pre-infection GR and lesion scores observed at PM after exposure to vNDV was weak and not different from zero. It was further observed that the chickens after infection with LaSota had higher GR compared to before infection (Table 2). This could suggest that these types of chickens, are tolerant to immune stress [38] and that during vaccination, the immune system do not drain nutrients into moderating the immune system thus allowing for weight gain. However, the challenging doses were same as the normal vaccination doses for ND and might not have affected the GR. Higher doses of the vaccine in the experiment could be stressful to the chickens as observed by Wang et al. [39] while using different doses of NDV vaccines and assessing the effects on the GR on broiler chickens. The chickens were exposed to the natural strains of the virus when they had non-protective antibodies titers for ND thus become prone to the infection with typical NDV clinical signs and lesions including weight loss, thus, the post-infection GR and post exposure GR are not correlated. The average lesion scores after PM

examination on the different organs indicated positive and moderate significant correlations ranging from 0.52 to 0.73 with highest correlation between the average lesion score and the lesions in the intestines similar to observation by [40].

From the research, males of the Tanzanian FRLC were more likely to die compared to females. These results are similar to findings by Gabriel et al. [41] where they recorded significantly higher mortalities in male broiler chickens from two to eight weeks of age. Female chicks were observed to have higher humoral and cell-mediated immune responses. On experimental infection with *E. coli*, NDV and Bovine serum albumin, females could reach peak antibody response 24-72 hours earlier than males. This could explain the higher incidence of mortality in the males than the females in this experiment. There were differences in survival among replicates, with better chances of survival for replicate 3 compared to replicates 1, 2 and 4. This could be explained by the fact that they were the youngest among then replicates at the time of exposure to the vNDV. The difference in NDV strains could also explain this difference as certain NDV strains in market birds could cause higher mortality than others.

Conclusions

There are no strong correlations between the traits at pre-infection GR, at post-infection GR and at post-exposure GR to virulent field strains of NDV in the FRLC. For some of the traits, there were significant negative correlations. There were significant differences among the chicken ecotypes for some traits and more clearly

with some clear differences among individual chickens within ecotypes due to the genetic admixtures in the chicken populations attributable to free ranging behavior that allows for random mating. The FRLC are highly outbreed and with significant variations even within the chicken populations, therefore, further work is needed to find and associate genetic elements with the significant phenotypic response in the chicken populations. This will best complement the existing phenotypic FRLC selection method being practiced by farmers and the chicken breeders. Our work has however contributed significantly into insight and understanding of correlated phenotypic traits that can be used for selection of chickens with reduced susceptibility to ND among the Tanzanian FRLC.

Declarations

Ethics approval and consent to participate

This study was approved by the research committee of the Sokoine University of Agriculture and the University of California Davis and it followed the Institutional Animal Care and Use Committee (IACUC) protocol of University of California Davis (# 20831) to conduct experiments

Consent for publication

Not applicable

Availability of data and material

The datasets supporting the conclusions of this article are available in the USAID Data Development Library (DDL) repository, and can be accessed publicly at the https://www.usaid.gov/development-data-library request.

Competing interests

The authors of this paper declare that they do not have any competing interests

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Authors' contributions

All authors read and approved the manuscript. JM analyzed the linear phenotype measurements and was the primary author of the manuscript, GC and EM were responsible for preparation of the experimental chickens, and data collection, MW conducted the structure analysis from the SNP genotypes and participated in all phases of the paper write up from conceptualization, analysis to the final write up, EA participated in the structure analysis, HZ is the principle investigator for the Genomics to Improve Poultry Innovation Lab, AM is the principle investigator for this study, PM, RM, HZ, JD, SL, TK and RG participated in the conceptualization of

the study and the review of the manuscript drafts before final submission to the journal.

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Table 4.1: Lesion score scale for the natural velogenic NDV exposure trial

Score	Description
0	Normal
0.5	Mild
1	Mild to moderate
2	Moderate
3	Moderate to severe
4	Severe

		Lesion score			
Statistic	**Post-exposure GR[4]**	**Trachea[5]**	**Proventriculus[6]**	**Cecal Tonsils[7]**	**Intestines[8]**
N[1]	632	244	244	244	244
Minimum	-85.7	0	0	0	0
Maximum	21.4	3	3	3	3
Mean[2]	-10.2	0.53	0.10	0.71	0.82
SD[3]	16.67	0.56	0.33	0.60	0.49
Heritability[2]±SE[5]	-	0(n.e)	0.05(0.05)	0.05(0.06)	0.03(0.06)

Table 4.2: Descriptive statistics of growth rate and lesion score in organs of the chickens after exposure to velogenic field strains of NDV

[1]Number of phenotype records, [2]arithmetic mean of phenotypic traits, [3]standard deviation, [4]growth rate after infection with velogenic field strains of NDV in g/day, [5,6,7,8] lesion scores in trachea, proventriculus, cecal tonsils and intestines respectively.

Table 4.3: Estimates (±SE) of phenotypic correlations among trait responses to lentogenic and velogenic NDV strains infection

	Pre-infection GR	**Post-infection GR**	**Antibody**	**Post-exposure GR**	**Average lesion score**	**Trachea**	**Proventriculus**	**Intestines**	**Tonsils**	**VL2dpi[4]**	**VL6dpi[4]**	**VCl[5]**
Pre-infection GR[1]		0.47 (0.03)	0.12 (0.04)	-0.10 (0.07)	0.09 (0.05)	0.02 (0.05)	0.07 (0.05)	0.07 (0.05)	0.06 (0.05)	-0.11 (0.06)	-0.17 (0.07)	0.06 (0.10)
Post-infection GR[1]			-0.002 (0.04)	-0.02 (0.06)	0.12 (0.04)	0.04 (0.04)	0.03 (0.04)	0.09 (0.04)	0.04 (0.04)	-0.16 (0.06)	-0.22 (0.06)	-0.12 (0.09)
Antibody				-0.02 (0.06)	-0.05 (0.05)	-0.02 (0.04)	-0.13 (0.04)	-0.008 (0.04)	0.04 (0.04)	0.1 (0.06)	0.04 (0.060	0.08 (0.09)
Post-exposure GR[2]					-0.002 (0.05)	-0.18 (0.04)	-0.28 (0.05)	0.07 (0.04)	0.20 (0.05)	-0.07 (0.09)	-0.11 (0.09)	-0.18 (0.13)
Average lesion score						0.58 (0.03)	0.52 (0.03)	0.73 (0.02)	0.52 (0.03)	-0.10 (0.07)	0.003 (0.06)	-0.07 (0.1)
Trachea							0.15 (0.04)	0.17 (0.04)	0.05 (0.04)	-0.02 (0.06)	0.02 (0.06)	0.02 (0.09)
Proventriculus								0.27 (0.03)	-0.03 (0.04)	-0.15 (0.06)	-0.01 (0.06)	-0.12 (0.1)
Intestines									0.23 (0.04)	-0.03 (0.06)	-0.002 (0.06)	-0.1 (0.1)
Tonsils										-0.03 (0.06)	-0.07 (0.05)	0.01 (0.09)

[1]Growth rate before and after infection with LaSota strain (g/day), [2]Growth rate after exposure to field strains of NDV, [4]log_{10} viral load at 2 and 6 days and antibody titer at 10 days after infection with LaSota, [5]Viral clearance

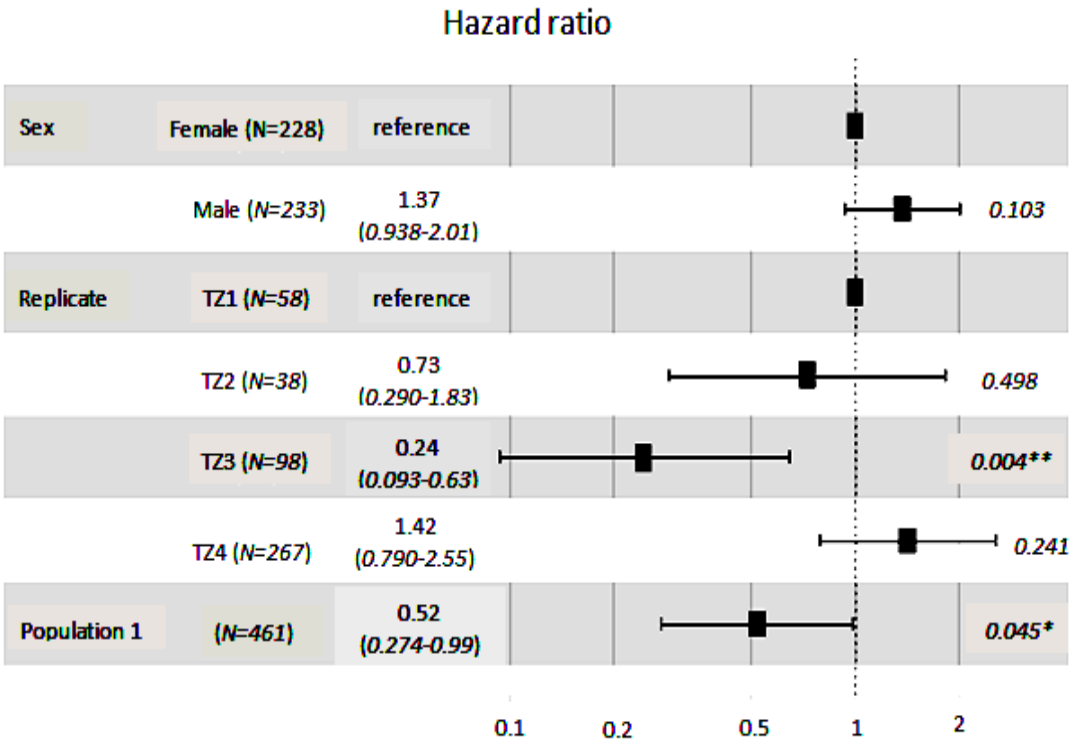

Figure 4.1: Survival analyses of the three FRLC ecotypes after exposure to virulent field strains of NDV. The sex, replicate number of the experiment and the population in which they belonged were significant covariates on survival of the chickens. N is the number of chickens, TZ is the number in which the chickens belonged to. Significance codes: 0 '*' 0.001 '**' 0.01 '*' 0.05 '.' 0.1 ' ' 1**

CHAPTER FIVE

5.0 GENERAL DISCUSSION

The objective of this study was to evaluate the innate resistance of Tanzanian free-range local chicken (FRLC) ecotypes to Newcastle disease (ND), a disease known to highly affect productivity in this type of chickens (Khatun *et al.*, 2018). FRLC are important assets in rural communities of Tanzania similar to many other developing countries of the world by supplying dietary protein for human nutrition in form of meat and eggs (MLFD, 2012; Pandhi, 2016). They are also important in other social events such as gifts, and religious ceremonies such as offerings to the deities. FRLC also suppliment family incomes through sale of chickens and chicken products (Gueye *et al.*, 1998) and also as savings and insurance (Besbes, 2009). FRLC production in Tanzania is commonly practiced under the extensive management and or backyard system (Sanka and Mbaga, 2014). This is the cheapest means of production of these types of chickens due to minimal input in production. However, under extensive management system, there are many challenges which affect productivity of the chickens and these include the frequent contact with reservoirs of infections like ND (Mwalusanya *et al.*, 2001; Mutayoba *et al.*, 2012).

The endemic presence of ND in the Tanzanian FRLC impacts on their productivity (Minga and Nkini, 1986; Melewas *et al.*, 1989; Yongolo *et al.*, 1996) thus threatening food security and contributes to deterioration of the national strategies for poverty alleviation through poultry industry. Control of the disease in the FRLC is a challenge due to the nature of the husbandry system in practice and the chicken flock

structures in villages. Vaccination is the only best way being practiced to control ND in FRLC in Tanzania (Yongolo *et al.,* 1996); however, it also faces many challenges such as poor supply to rural areas, lack of cold chains to handle the vaccines thus affecting vaccine viability and inappropriate knowledge in vaccine administration. Further, lack of breeding programs for any particular traits contributes to poor productivity of the FRLC. Selection of the chickens has mostly been dependent on nature and the fittest chickens have been surviving. Because of these, there are no pure breeds or lines of FRLC, instead there are ecotypes, generally, with different characteristics that can be used to distinguish them. Differences among the ecotypes could be attributable to adaptations to the different local eco-climatic zones in which they are found (Hartl, 1988; Barker, 1994). However, one has to be cautious with ecotype characteristics since in practice, people would move chickens from one place to another for instance in form of gifts or dowry or even individual perception where individuals buy and move chickens for breeding from one place to another, leading to panmictic populations of various proportions of genetic contributions.

Natural selection during ND outbreaks of ND leaves chickens which initiates new flocks after seasons of outbreaks. Researches have shown that some local chickens are naturally more resistant to some diseases than others (Gwakisa *et al.*, 1994; Mdegela *et al.*, 1998; Okoye *et al.,* 1999; Hassan *et al.,* 2004). This notion has left Tanzanian chicken keepers and breeders with uncertainties as to which chicken types are better for keeping than others and with resistance to diseases especially ND in order to minimize losses during outbreaks. Those questions led to the need to conduct this research aiming at; first to help to characterize some selected Tanzanian

FRLC ecotypes phenotypically and genotypically, secondly to analyze their immune responses during ND infection and also to correlate phenotypic traits that are important in assisting to select the FRLC for breeding purposes. Findings from this research would provide farmers and breeders with information for decision making during selection of chickens keep.

In this thesis, manuscript I demonstrate the use of some measurable phenotypic features of which can be used to characterize the FRLC ecotypes. It also describes the genetic population structure which can be used to describe the chickens further. Use of phenotypic measurable features have been used elsewhere, where, for instance, Olutogun *et al.* (2003) working on Zebu and Muturu cattle, found that the correlated body measurements could be used in selective breeding of the cattle for desired traits. In this study, genotyping and admixture analysis has revealed that there are two populations of chickens used in the experiments instead of three ecotypes as depicted by the phenotypic characterization. One of the populations (Population 1) consisted of higher genetic proportions of Ching'wekwe and Morogoro-medium while the other population (population 2) consists of higher genetic proportion of the Kuchi as compared to Ching'wekwe and Morogoro-medium as observed by Walugembe *et al.* (2019). These admixture of populations are most likely due to uncontrolled mating during free-ranging in the common feeding grounds and also during the transfer of chickens from one zone to another within the country similar to observations by Gondwe *et al.* (2002) in Malawi. Thus, caution should be taken during selection of chickens for breeding using phenotype

characteristics only. Additional genomic tools should be considered to complement the selection based on phenotypic features.

This study further investigated the immune response traits of the chickens during infection with NDV focusing on the viral loads at 2 and 6 days post infection, viral clearance rate and the expression of anti-NDV antibodies during infection as described in manuscript II. To the best of my knowledge, this is the first report in the evaluation of immune response to Newcastle disease in FRLCs using the viral loads and anti-NDV antibodies.

This research has also been able to demonstrate that Kuchi and Morogoro-medium can clear the NDV from their bodies faster than Ching'wekwe. It has also shown that they are better sero-converters than Ching'wekwe. These traits indicate that these chickens can better survive ND outbreaks compared to Ching'wekwe. However, caution should be taken when selecting for high immune response since the immunity and immune-modulatory molecules take up energy for other production values such as growth and egg production, thus, selecting for maximum immune response might not be the best option in the chicken production (Mashaly *et al.*, 2000; Klasing, 2004).

The research also indicates high growth rate in Kuchi than Morogoro-medium and least growth rate in Ching'wekwe contrast to the expectation that due to the high immune response in these two chicken types, they would have the lowest growth rates as explained by Mashaly *et al.* (2000) and Klasing (2004). The Moderate

immune response and growth rate in the Morogoro- medium was also evidenced by Msoffe *et al.* (1998) who described the wide spread of Morogoro-medium ecotype of the chickens in Tanzania compared to the other two ecotypes which are more regionally localized ecotypes, this being an indication of their survival fitness in case of infections.

This research went further to assess phenotypic traits before, during and after infection of the FRLC with LaSota strain of NDV and exposure to virulent field strains of NDV and to evaluate their relationships as explained in manuscript III. The research revealed that sex, ecotype and their interactions had effects on the FRLC phenotypic traits, findings similar to those of Ahn *et al.* (1995); Cherian *et al.* (1996); Jaturasitha *et al.* (2008); Zhao *et al.* (2009). From this research relationships among the traits before and after the infections have been shown in manuscript III. Highest correlations were observed between mean lesion scores and lesion scores in the trachea, intestines, provetriculus and cecal tonsils after infection with veloenic field strains of NDV. Highest mean lesion scores were recorded in the intestines and least in the proventriculus, probably due to differences in the tissues tropism by the viruses and availability of proteases in the tissues which are important for activating the virulent fusion protein (F-protein) of the viruses (Hussein *et al.*, 2019; Irshad *et al.*, 2019). It was expected that the different chicken ecotypes would have different responses during infections with NDV, thus varying in the severity of the lesion scores. Mean lesion scores in this study were high for Kuchi (0.79±0.06), and lowest for Ching'wekwe (0.71±0.04) with Morogoro-medium having a mean lesion score of (0.715±0.04).

Other researchers have indicated positive and significant correlations between live body weights and measurements of different body parts, where, the body measurements have been used to predict the body weights of animals. In cane rat for instance, Kolawole and Salako (2010) reported a positive relationship between live weight and body length and heart girth. In local fowls, Ige *et al.* (2007) reported positive phenotypic correlation between body weight and linear measurements, while Razuki *et al.* (2011) reported significant strain differences in body weight at different ages among breeds of broiler chickens.

5.1 Conclusions and recommendations

This thesis aimed at evaluating innate resistance to ND in FRLC in Tanzania and to provide guidance for selection in breeding programs for FRLC to improve productivity and livelihoods in rural areas. The study has contributed to the following body of existing knowledge;

- The study has provided basis for characterization of the FRLC using measurable phenotypic features and has also enlightened on the genetic population structure of the Tanzanian FRLC after genotyping. The thesis has brought an understanding that phenotypically, the chickens can be categorized into three distinct ecotypes as known from their physical features and that genetically the chickens are actually two populations with different proportions of admixture; Kuchi forming one population while Morogoro-medium and Ching'wekwe forming another population.
- This thesis has demonstrated the differences in immune response among Tanzanian FRLC ecotypes based on ND virus clearance rate and sero-conversion

during NDV infection, parameters which are important in selection of the Tanzanian FRLC

- Findings of this thesis have shown the effects of ND in the FRLC's performances through evaluation of growth rates at different times and with different strains of NDV as an indicator trait.
- This thesis has also shown relationships among phenotypic traits and responses during infection of the FRLC with LaSota a lentogenic strains of NDV commonly used as a vaccine against ND and virulent field strains of NDV.

5.2 Future research perspectives

Demand for chickens as a source of cheap and readily available animal protein in form of meat and eggs is growing. Demographically, Tanzania's population is increasing rapidly with an increasing demand for animal proteins. Rural communities may be more prone to deficiency of this main animal protein source due to challenges in production of the FRLC as explained. This thesis has worked with only three famous and most popular Tanzanian ecotypes because they are the most common chicken types in the country and which have previously researched on thus, have more information than others, however, there are many other FRLC ecotypes that have not been researched on. In view of this, efforts should be made to do similar researches using other ecotypes to improve the current knowledge in order to improve productivity of Tanzanian FRLC and livelihoods in the rural communities of the country.

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APPENDICES

Appendix 1: ELISA Test Procedure

All reagents must be allowed to come to 18-26°C before use. Mix reagents by gentle inverting or swirling.

1. Obtain antigen coated plate(s) and record the sample position.
2. Dispense 100µL of UNDILUTED Negative control (NC) into duplicate wells.
3. Dispense 100µL of UNDILUTED Positive control (PC) into duplicate wells.
4. Dispense 100µL of DILUTED samples into appropriate wells. Samples may be tested in duplicate wells, but a single well is acceptable.
5. Incubate for 30 minutes (±2 minutes) at 18-26°C
6. Remove the solution and wash each well with approximately 350 µL of distilled or deionized water 3-5 times. Avoid plate drying between plate washings and prior to the addition of the next reagent. Tap each plate onto absorbent material after the final wash to remove any residual wash fluid.
7. Dispense 100µL of conjugate into each well.
8. Incubate for 30 minutes (±2 minutes) at 18-26°C.
9. Repeat steps 6.
10. Dispense 100µL of TMB Substrate into each well.
11. Incubate for 15 minutes (1 minute) at 18-26C.
12. Dispense 100µL of stop solution into each well
13. Measure and record absorbance values at 650nm, A (650)
14. Calculation:

Controls

$$\begin{array}{l} 650 \\ \text{¿} \\ 650 \\ +NC\,2\,A(\text{¿}) \\ \text{¿} \\ 650 \\ \text{¿} \\ 650 \\ +PC\,2\,A(\text{¿}) \\ NC\,1\,A\,\text{¿} \\ NC\,x=\text{¿} \end{array}$$

Validity criteria

PCx-NCx>0.075 NCx≤0.150

For invalid assays, technique may be suspect, and the assay should be repeated following a thorough review of the package insert.

Samples

$$S/P = \frac{Sample\ Mean - NCx}{PCx - NCx}$$

Log_{10} Titer = 1.09(log_{10} S/P) + 3.36*

*Relates S/P at a 1:500 dilution to an end point titer

The presence of absence of antibody to NDV is determined by relating the (650) value of the unknown to the Positive Control mean. The positive control is standardized and represents significant antibody levels to NDV in chicken serum. The relative level of antibody in the sample is determined by calculating the sample to positive (S/P) ratio. End point titers are calculated using the equation descried above.

15. Interpretation:

Negative (S/P ≤0.20) Positive (S/P > 0.20)

A positive result (titer greater than 396) indicates vaccination or other exposure to NDV.

Appendix 2: Anti-NDV antibody titer processing template

Plate Identity

bird identification number

Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	+	6432	6432	6428	6428	7100	7100	7099	7099
B	8029	8029	9321	9321	2171	2171	2172	2172	7095	7095	6441	6441
C	6454	6454	6403	6403	6453	6453	6404	6404	6409	6409	6455	6455
D	6475	6475	9317	9317	6451	6451	9314	9314	6435	6435	7094	7094
E	6466	6466	6476	6476	6406	6406	6445	6445	6410	6410	8068	8068
F	7029	7029	9315	9315	6419	6419	7028	7028	6492	6492	6413	6413
G	6439	6439	6485	6485	2173	2173	8071	8071	8021	8021	6415	6415
H	8070	8070	6495	6495	7079	7079	7056	7056	8674	8674	Blank	Blank

Optical deasities

	1	2	3	4	5	6	7	8	9	10	11	12
A	0.05	0.05	0.3	0.234	0.889	0.849	0.957	0.915	0.277	0.33	0.532	0.48
B	0.45	0.362	0.113	0.109	0.381	0.398	0.203	0.21	0.218	0.223	1.366	1.347
C	0.964	0.901	0.114	0.094	0.326	0.336	0.375	0.373	0.96	1.011	0.595	0.603
D	0.492	0.45	0.39	0.343	0.889	0.912	0.836	0.792	0.651	0.677	0.523	0.482
E	0.465	0.492	0.252	0.255	0.164	0.177	0.512	0.506	0.194	0.239	0.263	0.22
F	0.549	0.516	0.285	0.256	0.233	0.218	0.226	0.232	0.262	0.249	0.322	0.276
G	0.888	0.843	0.214	0.189	0.179	0.185	0.611	0.63	0.327	0.314	0.264	0.235
H	0.278	0.281	0.19	0.137	1.083	1.142	0.12	0.123	0.45	0.461	0.044	0.044

negmean	pos mean	difference
0.05	0.267	0.217

Bird ecotypes

Plate 4	1	2	3	4	5	6	7	8	9	10	11	12
A	-	-	+	+	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Kuchi	Kuchi	Ching'wekwe	Ching'wekwe
B	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe
C	Moro Mid	Moro Mid	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe
D	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe
E	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe
F	Ching'wekwe	Ching'wekwe	Moro Mid	Moro Mid	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe
G	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe
H	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Ching'wekwe	Blank	Blank

bird number	ecotype	dpi	plate	well	OD1	OD2	mean	stdev	S/P ratio	titer	log titer
8029	Ching'wekwe	10	Plate 4	B1	0.450	0.362	0.406	0.062	1.641	3929.519	3.594339
6454	Moro Mid	10	Plate 4	C1	0.964	0.901	0.933	0.045	4.067	10570.31	4.024088
6475	Ching'wekwe	10	Plate 4	D1	0.492	0.450	0.471	0.030	1.940	4717.657	3.673726
6466	Ching'wekwe	10	Plate 4	E1	0.465	0.492	0.479	0.019	1.975	4809.338	3.682085
7029	Ching'wekwe	10	Plate 4	F1	0.549	0.516	0.533	0.023	2.224	5473.574	3.738271
6439	Ching'wekwe	10	Plate 4	G1	0.888	0.843	0.866	0.032	3.758	9698.644	3.986711
8070	Ching'wekwe	10	Plate 4	H1	0.278	0.281	0.280	0.002	1.058	2435.073	3.386512
9321	Ching'wekwe	10	Plate 4	B3	0.113	0.109	0.111	0.003	0.281	574.4714	2.759268
6403	Ching'wekwe	10	Plate 4	C3	0.114	0.094	0.104	0.014	0.249	503.0001	2.701568
9317	Ching'wekwe	10	Plate 4	D3	0.390	0.343	0.367	0.033	1.459	3456.736	3.538666
6476	Ching'wekwe	10	Plate 4	E3	0.252	0.255	0.254	0.002	0.938	2135.965	3.329594
9315	Moro Mid	10	Plate 4	F3	0.285	0.256	0.271	0.021	1.016	2331.172	3.367574
6485	Ching'wekwe	10	Plate 4	G3	0.214	0.189	0.202	0.018	0.698	1548.491	3.189909
6495	Ching'wekwe	10	Plate 4	H3	0.190	0.137	0.164	0.037	0.523	1130.328	3.053204
6432	Ching'wekwe	10	Plate 4	A5	0.889	0.849	0.869	0.028	3.774	9744.024	3.988738
2171	Ching'wekwe	10	Plate 4	B5	0.381	0.398	0.390	0.012	1.565	3731.421	3.571874
6453	Ching'wekwe	10	Plate 4	C5	0.326	0.336	0.331	0.007	1.295	3036.329	3.482349
6451	Ching'wekwe	10	Plate 4	D5	0.889	0.912	0.901	0.016	3.919	10153.22	4.006604
6406	Ching'wekwe	10	Plate 4	E5	0.164	0.177	0.171	0.009	0.555	1206.521	3.081535
6419	Ching'wekwe	10	Plate 4	F5	0.233	0.218	0.226	0.011	0.809	1817.695	3.259521
2173	Ching'wekwe	10	Plate 4	G5	0.179	0.185	0.182	0.004	0.608	1332.553	3.124684
7079	Ching'wekwe	10	Plate 4	H5	1.083	1.142	1.113	0.042	4.896	12940.69	4.111957
6428	Ching'wekwe	10	Plate 4	A7	0.957	0.915	0.936	0.030	4.083	10616.02	4.025962
2172	Ching'wekwe	10	Plate 4	B7	0.203	0.210	0.207	0.005	0.721	1604.278	3.20528
6404	Ching'wekwe	10	Plate 4	C7	0.375	0.373	0.374	0.001	1.493	3546.116	3.549753
9314	Ching'wekwe	10	Plate 4	D7	0.836	0.792	0.814	0.031	3.521	9032.972	3.955831
6445	Ching'wekwe	10	Plate 4	E7	0.512	0.506	0.509	0.004	2.115	5183.639	3.714635
7028	Ching'wekwe	10	Plate 4	F7	0.226	0.232	0.229	0.004	0.825	1857.243	3.268869
8071	Ching'wekwe	10	Plate 4	G7	0.611	0.630	0.621	0.013	2.629	6570.184	3.817578
7056	Ching'wekwe	10	Plate 4	H7	0.120	0.123	0.122	0.002	0.329	683.05	2.834452
7100	Kuchi	10	Plate 4	A9	0.277	0.330	0.304	0.037	1.168	2713.906	3.433595
7095	Ching'wekwe	10	Plate 4	B9	0.218	0.223	0.221	0.004	0.786	1761.321	3.245838
6409	Ching'wekwe	10	Plate 4	C9	0.960	1.011	0.986	0.036	4.311	11264.1	4.051697
6435	Ching'wekwe	10	Plate 4	D9	0.651	0.677	0.664	0.018	2.829	7118.073	3.852362
6410	Ching'wekwe	10	Plate 4	E9	0.194	0.239	0.217	0.032	0.767	1716.328	3.2346
6492	Ching'wekwe	10	Plate 4	F9	0.262	0.249	0.256	0.009	0.947	2158.857	3.334224
8021	Ching'wekwe	10	Plate 4	G9	0.327	0.314	0.321	0.009	1.247	2912.871	3.464321
8674	Ching'wekwe	10	Plate 4	H9	0.450	0.461	0.456	0.008	1.869	4528.652	3.655969
7099	Ching'wekwe	10	Plate 4	A11	0.532	0.480	0.506	0.037	2.101	5146.72	3.711531
6441	Ching'wekwe	10	Plate 4	B11	1.366	1.347	1.357	0.013	6.021	16211.31	4.209818
6455	Ching'wekwe	10	Plate 4	C11	0.595	0.603	0.599	0.006	2.530	6300.757	3.799393
7094	Ching'wekwe	10	Plate 4	D11	0.523	0.482	0.503	0.029	2.085	5103.677	3.707883
8068	Ching'wekwe	10	Plate 4	E11	0.263	0.220	0.242	0.030	0.882	1999.046	3.300823
6413	Ching'wekwe	10	Plate 4	F11	0.322	0.276	0.299	0.033	1.147	2661.437	3.425116
6415	Ching'wekwe	10	Plate 4	G11	0.264	0.235	0.250	0.021	0.919	2090.242	3.320197
Blank	Blank	10	Plate 4	F12	0.044	0.044	0.044	0.000	-0.028	1	0

Appendix 3: NDV RNA isolation with MagMAX-96 Kit and Protocol

Purpose: Isolate RNA from chicken tears. The isolated RNA samples generated from this protocol will be used in qPCR to determine viral titer.

Lysis/Binding Solution (with Isopropanol added)	
RNA binding beads	Magnetic beads that bind RNA
Nuclease Free Water	
Wash Solution 1 (with Isopropanol added)	
Wash Solution 2 (with ethanol added)	
Elution Buffer	
Reagents that need to be Thawed out:	
Carrier RNA	Improves sample RNA binding efficiency to beads
Xeno RNA	Acts as a positive control and indicates the quality of isolation and qPCR prep after qPCR
Liquid Binding Enhancer	
Tear samples	

Outline of the steps:

1. Thaw Samples
2. Clean everything with anti-RNAse solution
3. Make Lysis/Binding Solution Master Mix:
    a. It is not necessary to add xeno RNA to every sample. Add xeno to about 25% of samples.
    b. This will mean you will make 2 Lysis/Binding Solution Master Mixes: one will be made with xeno RNA, and one will be made with RNAse-free water in place of xeno

4. Make Bead Mix
5. Combine Lysis/Binding Solution and samples (IMPORTANT: Record which samples are mixed with solution containing xeno)
6. Add Bead Mix.
7. Capture beads on magnetic stand. Remove Liquid, Take off Magnetic Stand
8. Wash with solution 1
9. Wash with solution 1
10. Wash with solution 2
11. Wash with solution 2
12. Dry beads thoroughly
13. Add elution buffer
14. Collect and save elution buffer
15. Analyze samples with Nanodrop

Appendix 4: **qPCR-Viral RNA extracted from Tears**

All reagents from Life Technologies

1. Plan the reactions
    a. Include Positive control (25X NDV Control DNA)
    b. Include no sample control reaction which contains Nuclease free water in place of RNA sample
2. Prepare standard NDV samples for generating a standard curve (provided by Thermo Fischer Scientific)
    a. Make a serial dilution of the NDV standard sample (10^8 virus titer) from 10^2 to 10^5
    b. Keep 10^2, 10^3, 10^4 and 10^5 tubes in ice for using as standard samples in the following qPCR
    c. Prepare to have one standard curve for each PCR plate
3. Prepare qPCR master mix
4. Assemble the qPCR master mix on ice using the following table. Include a 10% extra to ensure that you have enough master mix for all the samples

Component	**Per reaction**	**X reactions +10%**
2x qRT-PCR buffer	6.25µl	
25x qRT-PCR Enzyme mix	0.5µl	
25x qRT-PCR Primer probe mix	0.5µl	
Total Volume	7.25µl	

5. Place the prepared master on ice until it is needed
6. Distribute qRT-PCR master mix

7. Distribute 7.25µl of qRT PCR master mix to the wells of the PCR plate

8. Add sample to each well (2 technicla replicate s per sample)

9. Add 5.25µl of sample: the final reaction volume is 12.5µl

10. Program the real-time PCR machine

Use the parameters provided in the following tables. The 2X qRT-PCR Buffer contains ROX™ dye

The reaction volume is 12.5 µl

Target	Reporter	Quencher
NDV	FAM™	None
Xeno™ RNA Control	VIC™	None

Thermocycler settings:

	Stage	Reps	Temperature	Time
Reverse transcription	1	1	48°C	10 min
Enzyme activation/Template denaturation	2	1	95°C	10 min
Amplification	3	40	95°C	15 sec
			60°C	45 sec

11. Run the real-time thermocycler program and collect data

12. Analyze the data

a Generate the standard curve using the CT values pf each standard sample and get the equation.

b Calculate the viral load for each sample by the equation

Appendix 5: DNA Extraction from FTA cards

DNA Extraction from FTA cards

1. Cut out a small 3mm X 3mm square from the dried portions of blood on the using either a razor blade, scalpel blade or hole puncher. Be sure to clean the blade between samples to produce contamination.
2. Place the cut out into a 1.5ml tube with 500µl of sterile H_2O and vortex the sample ~ 10 times using a 1 second pulses.
3. Carefully transfer the cut out to a 0.2ml PCR tube with 30 µl of a sterile H_2O. Attempt to minimize the amount of water transferred with the cut out, as it will carry over salts and contaminants
4. Quick spin the PCR tubes and incubate at 98C for 30 minutes in a PCR machine with a heated lid
5. Prepare 1.5ml tubes and label with **first letter of country, trial #, ID#, and DNA** on both lid and side of tube.
6. After incubation vortex, the sample 60 times with 1 second pulses and quick spin.
7. Pipette supernatant into new 1.5ml tubes.
8. Add 1/10th the volume (3µl) of 3M Sodium Acetate.
9. Add 2X volume (~ 60 µl) of 100% Ethanol and invert the sample 20-30 times
10. Incubate on ice or at -20C for 15 minutes or longer(overnight)
11. Centrifuge for 30 minutes at room temperature at maximum speed
12. Discard supernatant by pouring out and add 500µl of cold 80% Ethanol.
13. Mix and centrifuge for 10 minutes.
14. Discard supernatant by pouring out, spin and remove by pipetting any residual ethanol.
15. Air dry for at least 10 minutes (more if needed to dry all ethanol).
16. Resuspended in 30µl of H2O

Quality Checking DNA:

1. DNA concentration in above 100ng/µl
2. Expected Ratios from nanodrop:
    a. 260/280 ~ 1.80
    b. 260/230 ~ 2.00

Appendix 6: PROTOCOL: Natural NDV Exposure Trial using Non-vaccinated Birds

- Birds
    - Seeder birds: hatch seeder birds for trials 2 weeks before the test birds hatch.

- Trial birds: sample size of 400-500 birds per replicate – a total of 3 replicates required.
- Raise trial birds to 4 weeks before NDV exposure (to make sure that maternal antibody levels have waned).

- Natural Challenge
  - Scout for sick birds at the bird market or village farms regularly and start 2 weeks before trial.
  - For 400 birds in a trial, obtain 5 sick market birds to mix with 50 seeder birds.
  - Collect 2 oro/cloacal swabs from each of the 5 sick market birds for NDV characterization.
    - Use a sterile, polyester-tipped swab with aluminum or plastic shaft.
    - Rub the swab tip gently but thoroughly against the back of the bird's throat and choanal slit.
    - Using the same swab, gently insert the swab into the bird's cloacal cavity. Gently rotate the swab back and forth 2-5 times.
    - Place the swab tip into a 2 ml cryovial with 0.5ml RNAlater.
    - Use ethanol-wiped, flame sterilized scissors to cut shaft of the swab above the tip or snap the plastic shaft by lifting the swab a bit above the bottom of the vial and then snapping it to ensure the swab shaft will not block the cap.
    - Store the crovials in a cooler with ice packs and transfer to -80°C freezer ASAP.
  - Using one of the oro/cloacal swabs, confirm velogenic NDV infection in the sick market birds by performing diagnostic qRT-PCR. Also, rule out infection with avian influenza with the rapid snap test.
  - Put the velogenic NDV positive birds into a pen with the seeder birds.
  - Monitor twice a day the seeder birds for clinical signs consistent with velogenic NDV (drowsiness, ruffled feathers, diarrhea, mucous exudates, severe depression, cloacal eversion to look for petechiae).
  - Collect 1 oro/cloacal swab from 5 seeder birds using the technique described above.
  - Once there are 2-3 seeder birds with clinical signs consistent with velogenic NDV, immediately introduce the seeder birds into the pen with the trial birds (birds shed the highest amount of virus at day 2).
- Sample/data collection

- 2 oro/cloacal swabs from each sick market bird for NDV characterization. Use the technique described above to collect 2 swabs from each bird.
- 1 oro/cloacal swab from 5 seeder birds for NDV characterization using the technique described above.
- Blood for FTA cards – day 0 dpi (day 0 = before introducing seeders)
- Blood for serum – day 0 dpi, day 7dpi and end of experiment (store in -20°C) for natural antibodies and innate levels of cytokines (day 0), and anti-NDV antibodies (day 0 and day 7 and end of the trial).
- Monitor mortalities twice a day (early morning and afternoon) to make sure that mortalities are captured immediately. High mortality is anticipated, so make sure to have adequate logistical arrangements for monitoring.
- Body weight: 0, 6, 10 dpi and at the time of euthanasia in 21 dpi (trial termination).
- Tears at 2 dpi, 6 dpi, 10 dpi (store at -80°C) for NDV load/viral clearance.
- 1 oro/cloacal swab at 2 dpi, 4 dpi, and 6 dpi for NDV shedding and clearance. Use the technique described above to collect 1 swab from each bird.
- Lesion scores on birds that die during the trial and that survive until study endpoint.

- Study end point = 21 dpi

  Major activities

DPI	Activity	Comments
0	Oro/cloacal swabs	Collect 2 swabs from each sick market bird and store at -80°C for NDV characterization.
0	Body weight	
0	Blood for FTA cards	To be shipped to US for DNA isolation and genotyping
0	Blood for serum	Store at -20°C for natural antibodies and innate levels of cytokines (day 0), and anti-NDV antibodies
0	Oro/cloacal swabs	Collect 1 swab from 5 seeder birds and store at -80°C for NDV characterization.
2	Tears	Store at -80°C for NDV load/viral clearance assay
2	Oro/cloacal swabs	Collect 1 swab from all birds and store at -80°C for NDV shedding/viral clearance assay
4	Oro/cloacal swabs	Collect 1 swab from all birds and store at -80°C for NDV shedding/viral clearance assay.

6	Body weight	
6	Tears	Store at -80°C for NDV load/viral clearance assay
6	Oro/cloacal swabs	Collect 1 swab from all birds and store at -80°C for NDV shedding/viral clearance assay.
7	Blood for serum	Store at -20°C for ELISA to determine anti-NDV antibodies
10	Body weight	
10	Tears	Store at -80°C for NDV load/viral clearance assay
End	Body weight, tears and serum	
Each day	Mortality/survival time	Monitor mortalities twice a day (early morning and afternoon) to make sure that mortalities are captured immediately.
Each day	Post-mortem examinations	Perform post-mortem examinations on all dead chickens each day. Collect tissues (lungs, trachea, brain, cecal tonsils, spleen, proventriculus, intestines) from a sample (about 20%) of the dead birds each day (with equal representation from each ecotype if possible) and preserve in 10% formalin (1:10 ratio of tissue to formalin) for histopathology. Record lesion scores on all dead birds for hemorrhages in trachea, proventriculus, intestines, and caecal tonsils. Record whether there are lesions in other tissues. Record the person who did the scoring.
End	Post-mortem examinations	Euthanize (CO_2) and perform post-mortem on all surviving birds. Record lesion scores. Collect tissues (lungs, trachea, brain, cecal tonsils, spleen, proventriculus, intestines) in 10% formalin for histopathology.

- Pathology
  - Birds that die during challenge:
    - ✓ Perform post-mortem examinations on all dead chickens each day. Collect tissues (lungs, trachea, brain, cecal tonsils, spleen, proventriculus, intestines) from a sample (about 20%) of the dead birds each day (with equal representation from

each ecotype if possible) and preserve in 10% formalin (1:10 ratio of tissue to formalin) for histopathology.

- ✓ Record lesion scores on all dead birds for hemorrhages in trachea, proventriculus, intestines, and caecal tonsils. Record whether there are lesions in other tissues. Record the person who did the scoring.

Score	Severity	Description
0	No lesions	No lesions
0.5	Mild	Inflammation/Mucous/froth/Mild/slightly/weak/petechiation
1	Moderate	Haemorrhagic lesion / +
2	Severe	Extensive haemorrhages / ++
3	Very Severe	Severe haemorrhages / +++
4	Extremely severe	Very severe haemorrhages / ++++

- o Birds that survive to the end of the challenge:
  - ✓ Weigh and collect tears and serum samples from all surviving birds.
  - ✓ Euthanize (with CO_2) and perform post-mortem examinations on all surviving chickens at the end of the trial.
  - ✓ Collect tissues (lungs, trachea, brain, cecal tonsils, spleen, proventriculus, intestines) in 10% formalin for histopathology.
  - ✓ Record lesion scores for hemorrhages in trachea, proventriculus, intestines, and cecal tonsils. Record whether there are lesions in other tissues. Record the person who did the scoring.