EPIDEMIOLOGY OF NEWCASTLE DISEASE IN BACKYARD CHICKENS REARING SYSTEM IN IRINGA RURAL DISTRICT, TANZANIA

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DISSERTATION SUBMITTED IN PARTIAL FULFILMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE IN ONE HEALTH MOLECULAR BIOLOGY OF SOKOINE UNIVERSITY OF AGRICULTURE.MOROGORO, TANZANIA.

2020

ABSTRACT

Newcastle disease (ND) outbreaks have been occurring in Iringa backyard chickens and causing high mortalities leading to severe economic losses to rural households. The study aimed at ascertain the epidemiological status of Newcastle disease (ND) in Iringa rural district focusing on determining risk factors of the disease, prevalence of the disease and genomic characteristics of NDV the etiology of the disease. A total of 250 structured questionnaires were subjected to respondents to assess the knowledge and practices with regard to ND. The results of questionnaire survey depict that all respondents (100%) were aware with the disease and 91.2% had experienced the problem in their backyard chickens. Also 69.6 % of the respondents were aware about the presence of vaccine against the disease. Most respondents (52.2%) were following the correct vaccination regime by vaccinating three times a year, while 29.6% of the respondents were vaccinating their chickens only once and 15.2% reported to have never vaccinated their chickens. The awareness with disease and proper follow -up of vaccination regime by the chicken keepers might be the contributing factor to the low prevalence of the disease. Majority of the respondents (68%) kept chickens only in the backyards, while 32% of respondents owned other birds such as ducks, pigeons, geese and quills. About 60% of the respondents reported that their chickens were interacting with wild birds while 40% of the respondents reported that their chickens were not interacting with wild birds. Other birds apart from chickens including wild birds act as reservoir for the disease and maintain the circulation of the virus in backyard chickens. A total of 321 swabs and 63 tissue samples were randomly collected from backyard chickens and virus detected by conventional reverse transcription polymerase chain reaction (RT-PCR). Two chickens out of 384 were found positive for NDV, therefore the detection rate of disease was 0.01%. The low detection rate could be due to most of the

chickens were the survivors of a preceding outbreak. Partial F-gene sequencing using cycle dideoxy nucleotide sequencer and phylogenetic analysis using MEGA X software shows that the circulating NDV strain in the backyard chickens in Iringa belong to genotype VII and is similar to Mozambican isolates of 2011 and 2012. Moreover, the isolate belongs to a different genotype with currently used vaccines. This study has shown that NDV is still a threat in most backyard chickens as it causes massive death of chickens and therefore mass education is essential in order to reduce the burden. Also, regular molecular epidemiological studies on the characteristics of the virus will help to improve vaccine and vaccination measures with regard to existing circulating strains to end the problem in backyard chickens rearing communities.

DECLARATION

I, Isaya Musa Kibasa, do hereby declare to the Senate of Sokoine University of Agriculture that this dissertation is my own original work done within the period of registration and that it has neither been submitted nor being concurrently submitted in any other institution.

_____ _____

Isaya Musa Kibasa Date

(MSc. OHMB Candidate)

The above declaration is hereby confirmed by;

_____ _____

Dr. Augustino Chengula Date

(Supervisor)

_____ _____

Prof. Gerald Misinzo Date

(Supervisor)

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ACKNOWLEDGEMENTS

First and fore most I would like to thank God who gave me strength from the beginning of my studies at Sokoine University of Agriculture.

Secondly, I would like to express my heartfelt thanks to my supervisors Dr. Augustino Chengula and Professor Gerald Misinzo for their closest and tireless support without absenting themselves in each stage of doing my research. Let God protect them and stay blessed to continue with such a spirit to others.

I would like to extend my thanks to the government of the United Republic of Tanzania and the World Bank through the SACIDS Africa Centre of Excellence for Infectious Diseases for funding my studies. I wouldn't make it without their support as they made possible for me to reach this destination smoothly without obstacles.

I am also indebted to all chicken keepers in all wards visited and Government officers who paved the way for me to meet my needs without facing any difficulties.

Lastly but not least I highly acknowledge the excellent technical assistance from Ms. Mariam Makange during laboratory analysis.

DEDICATION

To my mother, wife and my daughter Charity and my son Charm

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LIST OF ABBREVIATIONS

APMV	Avian paramyxovirus
F	Fusion protein
g	Gravity
HA	Hemagglutination assay
HI	Hemagglutination inhibition test
HN	Hemagglutinin neuraminidase
ICPI	Intracerebral pathogenicity index
IVPI	Intra venous pathogenicity index
NCBI	National center for Biotechnology Information
L	RNA dependent RNA polymerase
M	Matrix protein
MDT	Mean death time
MgCL	Magnesium chloride
ND	Newcastle disease
NDV	Newcastle disease virus
NDV-F	Newcastle disease virus forward primer
NDV-R	Newcastle disease virus reverse primer
NP	Nucleoprotein
OIE	Office International des Epizooties
ORF	Open reading frame
P	Phosphoprotein
PCR	Polymerase chain reaction
PCR	Polymerase chain reaction
RNA	Ribonucleic acid
RT-PCR	Reverse transcription polymerase chain reaction
SACIDS	Southern African Centre for Infectious Disease Surveillance
SUA	Sokoine University of Agriculture
UV	Ultraviolet
˚ C	Percentage
µM	Micromolar
µl	Microliter

CHAPTER ONE

1.0 INTRODUCTION

1.1 Background Information

Newcastle disease (ND) is caused by a virus belonging to the genus Avulavirus within the family *Paramyxoviridae* in the order *Mononegavirales* (Alexander, 1998). Causative agent of ND is Newcastle disease virus (NDV), an enveloped, single stranded negative-sense RNA virus whose genome size is approximately 15 kilo bases. The genome of NDV has six open reading frames (ORFs) which encode for six major structural proteins, namely, nucleoprotein (NP), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin-neuraminidase (HN) and the RNA-dependent RNA polymerase (L) (Ganar *et al*., 2014).

Analysis of nucleotide sequences of the F gene classify the NDV into two classes: class I and II. Most of class I viruses are lentogenic and have been isolated from water fowl, shorebirds and domestic poultry and class II virus are velogenic and have been isolated from domestic poultry and wild birds and are the one responsible for outbreak of disease and hence causing great economic losses to poultry industry (Fan *et al*., 2015).

Newcastle disease is a highly contagious disease transmitted through infected dropping and through respiratory discharges from infected chickens to healthy chickens (Li *et al*., 2009). The disease is characterized by labored breathing and central nervous signs such as paralysis or twisted necks, associated with drop in eggs production by 30% to 50% or more. Eggs may have thin shells and without shells. In vaccinated chicken flocks, clinical signs may be difficult to find (Intervet, 1972).

Many reports and studies (Bell *et al.,* 1990; McBride *et al.,* 1991) suggest a continuous presence of NDV in village chicken's populations. Some of the risk factors that have been associated with the maintenance of NDV include: carrier chickens, village poultry population dynamics, other poultry species, wild birds and heterogeneity (Awan *et al.*, 1994). Although clinically diseased chickens are the most important hosts for NDV, latently infected birds and survivors of natural infection, which still harbour the virus, may also act as reservoirs (Awan *et al.,* 1994).

Despite the fact that the ND causes a great loss to farmer practicing backyard chickens rearing system in most rural setting in Tanzania, there is scant information about the genomic epidemiology of NDV in Iringa. Therefore, the aim of the study was to conduct a molecular epidemiological study of NDV in Iringa Rural District.

1.2 Problem Statement and Justification of Study

Most farmers in Iringa rural keep their chickens in backyard system as a source of income and food but they are likely losing their chickens because of diseases such as ND and the incidence of death of chickens is high during dry seasons. Despite vaccination, ND outbreaks sometimes still occur. It is not known why vaccinated chickens succumb to ND. Selim *et al.* (2018) reported on the mutation which occurred in the fusion protein of NDV in Egypt and affecting its configuration and eventually leading to failure of vaccine protection. However, there is scant information on the genetic characteristics of NDV in Iringa which could help in the proper control of the disease.

Therefore, the present study was conducted in order to understand the epidemiology and the genetic characteristic of NDV. This will help to provide baseline information on

epidemiological status of NDV in Iringa rural for the appropriate measures of the disease and hence reduce the burden to farmers of losing their chickens.

1.3 Research Questions

i. What is the prevalence or detection rate of Newcastle diseases in Iringa Rural District?
ii. What is the genotype of Newcastle disease virus circulating in backyard chickens in Iringa Rural District?
iii. What are the risk factors of Newcastle diseases in backyard chickens in Iringa Rural District?

1.4 Research Objectives

1.4.1 General objective

The overall objective of this study was to ascertain the molecular epidemiological status of NDV circulating in backyard chickens in Iringa Rural District, Tanzania.

1.4.2 Specific objectives

i. To determine the prevalence of Newcastle disease in backyard chickens in Iringa Rural District by reverse transcription polymerase chain reaction (RT-PCR),
ii. To genetically characterize the NDV circulating in backyard chickens in Iringa Rural District using molecular techniques, and
iii. To determine the risk factors of Newcastle disease in the backyard chickens in Iringa Rural District.

CHAPTER TWO

2.0 LITERATURE REVIEW

2.1 Newcastle Disease

Newcastle disease is a contagious viral disease caused by avian paramyxovirus serotype 1 (APMV-1). There are other eight serotype namely APMV-2 to APMV-9 and most of these eight serotypes are found in reservoir avian species. However, only APMV-2 and APMV-3 viruses have made a significant disease and economic impact on poultry production (Alexander, 2000).

2.2 Species Affected and Susceptibility

Almost 8000 species of birds seem to be highly susceptible to virulent APMV-1 including chickens, turkeys, pheasants, pigeon, quails and guinea fowl (Kaleta and Baldauf, 1988). Waterfowl such as ducks and geese may be infected but show few or no clinical signs even to the virulent strains of chickens (Liu *et al*., 2007; Yongolo *et al*., 2002).

2.3 Transmission

Newcastle disease is transmitted from infected chickens to healthy chickens mainly through direct contact of discharges (Li *et al*., 2009). The disease is often mechanically spread by vaccination and debeaking team, manure haulers, feed delivery personnel,

poultry buyers, egg service people and poultry farm owners and employees, where virus-bearing materials can be picked up on shoes, clothing or equipment and carried from an infected flock to a healthy one (Solomon, 2012). The experimental studies proved that the aerosol exposed chicken with the virus was able to be contracted with the virus and able to shed it indicating that airborne transmission is possible (Li *et al*., 2009).

According to Chen and Wang (2002), egg- associated vertical transmission with virulent strains is possible. However, it is not common and usually the embryo dies unless if the titer of virus is low. The fecal oral route is the main for the spread of the virus from infected birds to healthy bird (Alexander, 1988).

2.4 Diagnosis

Newcastle disease is diagnosed based on the clinical signs mainly high morbidity and mortality and confirmed in the laboratory by isolating the virus from the tissues of dead chickens and swabs of the live chickens (Solomon, 2012). Virus isolation is done by inoculating the virus from samples into 9 to11-day-old embryonated chicken eggs and confirming the presence of the virus in the allantoic fluid from dead embryos using hemagglutination assay (HA) and hemagglutination inhibition (HI) assay with monospecific antiserum to APMV-1. Molecular detection of NDV-RNA from tissues or swabs is performed by using RT-PCR that specifically targets NDV- RNA amplification (Ewies *et al*., 2017).

2.5 Zoonotic/Public Health Significance

Newcastle disease infection has zoonotic implication in human as it causes conjunctivitis upon contacts. However, there are reports of general infection resulting in chilling,

headache and fever without conjunctivitis due to direct contact with the virus (Alexander, 1988).

2.6 Virion Structure

When viewed under electronic microscope, NDV appears pleomorphic. Most of the virion are roughly spherical with diameters around 100 to 500 nm. This virion is covered with lipid bilayer membrane (Yusoff and Wen, 2001), attached with hemagglutinin neuraminidase (HN) and the fusion protein (F) which appears like small projections on the surface of viral envelope. These HN and F glycoprotein on the surface are the principal antigen that elicit protective or defensive immune response (Seal *et al.*, 2000).

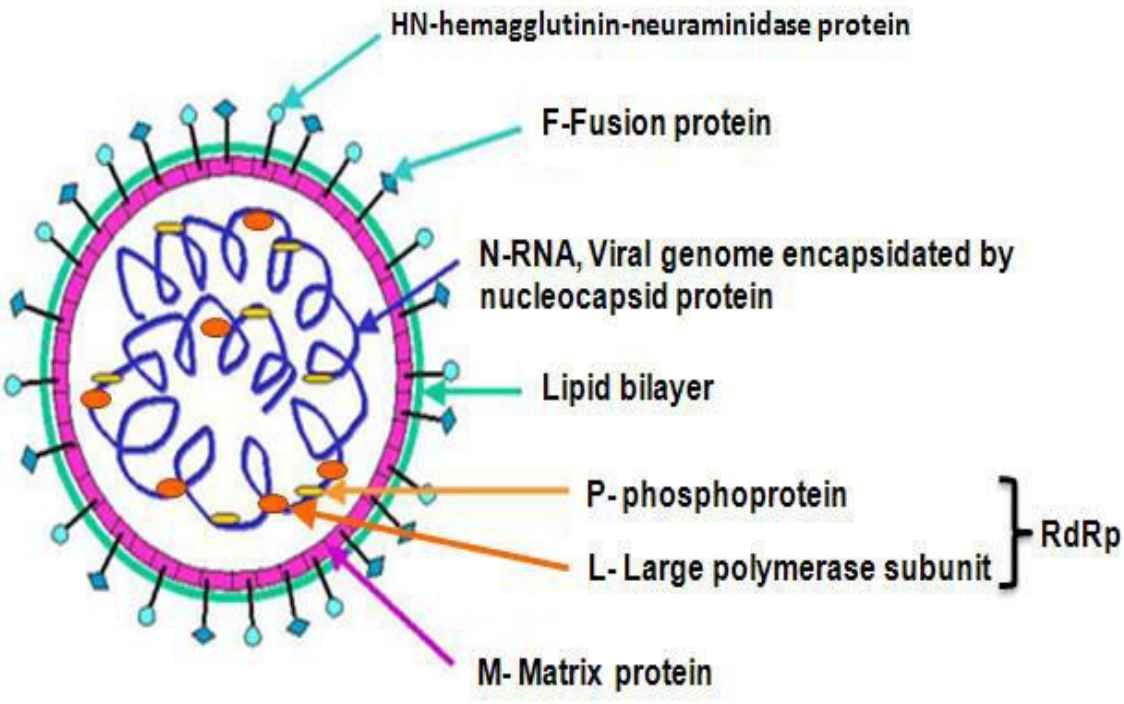

Figure 1: Newcastle disease virion structure

Source: Seal *et al.* (2000).

2.7 Virus Characterization and Strains

2.7.1 Biological characterization

Newcastle disease virus can either be categorized as lentogenic, mesogenic and velogenic strains basing on its virulence and pathogenicity. The pathogenicity of the newly isolated virus can be assessed by three methods where by the first is known as mean death time (MDT) in chicken embryos. In this method the lethal dose is calculated by time it will take to kill the egg embryo and it is estimated to be under 60 hours for velogenic, 60 to 90 hours for mesogenic and more than 90 hours for lentogenic (OIE, 2009).

The second method is the intracerebral pathogenicity index (ICPI), where virus rich fresh allantoic fluid is inoculated into the brain of ten-day-old chicks from a specific pathogen-free flock. The birds are examined at 24-hour intervals for eight days and graded zero if normal, one if sick and two if dead. The index is the mean score per bird per observation over the ten-day period. The most virulent viruses give ICPI values approaching the maximum score of 2.0, while lentogenic viruses give values of, or close to 0.0 (OIE, 2009).

The third method is the intravenous pathogenicity index (IVPI*)* where by fresh infective allantoic fluid with HA titer of >24 is diluted 1:10 in sterile isotonic saline and 0.1ml of the diluted virus is injected intravenously into 6-week-old SPF chickens. Birds are examined at 24-hour interval for 10 days and scored at each observation; 0 if normal, 1 if sick, 2 if paralyzed or showing other nervous signs and 3 if dead. The mean IVPI is the score per bird per observation over 10-day period. Lentogenic strains and some mesogenic strains will have IVPI values of 0, whereas the indices for virulent strains will approach 3.0 (OIE, 2009).

2.7.2 Molecular basis/determinant of virulence

Apart from the conventional biological methods of characterization of NDV strains, sequence analysis of the F protein cleavage site has been used to predict potential pathogenicity of NDV (Panda *et al.*, 2004). The primary molecular determinant for ND pathogenicity is the amino acid sequence at the Fusion protein cleavage site (F0) and the ease with which cellular proteases cleave the fusion protein (de Leeuw *et al*., 2005; Gotoh *et al*., 1992).

The F glycoprotein inactive (F0) is proteolytically cleaved at peptide bond between residue 116 and 117 making two active polypeptides which are F1 and F2 (Liu *et al*., 2003). Virulent NDV isolates commonly have dibasic amino acids flanking a glutamine residue (^{112}RRQR/KR^{116}) at the carboxyl terminus of the F1 polypeptide. Avirulent isolates have a neutral amino acid in the place of basic arginine residues (^{112}GR/KQG/SR^{116}). Furthermore, the amino acid at position 117 of the F2 polypeptide N-terminus is phenylalanine or leucine residue in virulent and avirulent NDV strains respectively (Peroulis-Kourtis *et al.*, 2002).

CHAPTER THREE

3.0 MATERIALS AND METHODS

3.1 Study Area

The present study was conducted in Iringa rural district in five wards namely Luhota, Magulilwa, Mgama, Lyamugungwe and Mseke (Fig. 2). The district bordered to the north by Mpwapwa district in Dodoma region, to the east by Kilolo district and encircles Iringa Urban district, to the south by the Mufindi district, to the southwest by Mbarali district in Mbeya region and to the northwest by Manyoni district in Singida region.

Iringa rural district is estimated to have a total of 245 623 general populations according to Tanzania National Bureau of Statistics website report of year 2002. The climate is warm with average annual temperature of around 19.1°C and rainfall of 690 mm.

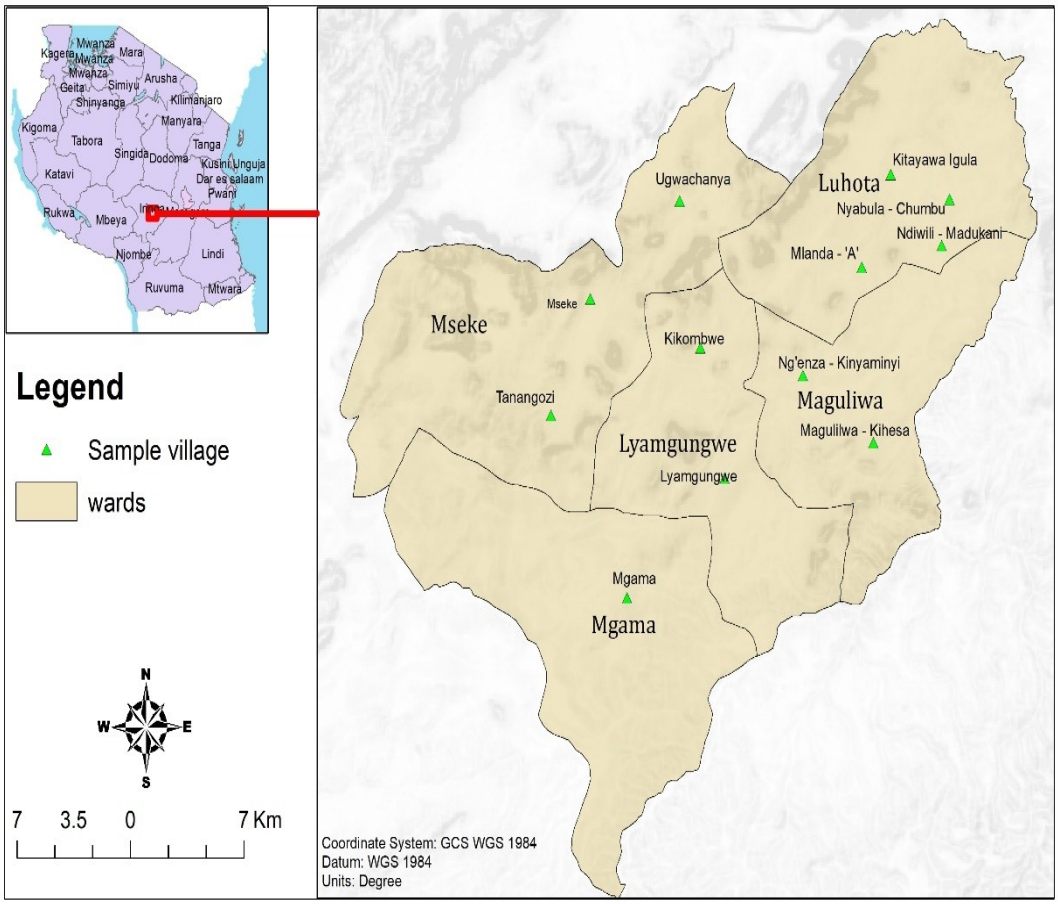

Figure 2: **Map of Iringa rural district to show the geographical location of study areas**

Source: Field Data (2020)

3.2 Study Design

The present study was a cross sectional study that was conducted to assess knowledge, attitudes, practices and estimate the prevalence of ND in the study location. The study population involved randomly selected farmers who kept chickens in their backyards.

3.3 Sample Size Estimation

The sample size was calculated by using the following formula (Naing *et al*., 2006) $n=Z^2p(1-p)/L^2$. Where n defined as sample size, Z defined as confidence interval =

(1.96), P defined as prevalence of disease =50% (for unknown prevalence), L defined by expected error =0.05, Sample size =1.96^2*0.5(1-0.5)/0.05^2 = 384 chickens.

3.4 Sampling of the Chickens

Tracheal swabs (321) were collected from the selected vaccinated and unvaccinated live chickens. In addition, tissues (63) such as cecal tonsils, proventriculus, trachea, brain and liver were collected from dead chickens which showed pathognomonic lesions during postmortem examination. All samples were collected in a total of 250 households where by the number of samples per household were unevenly (1 or 2) with regard to number of chickens and health status of chickens. Samples were collected into 95% ethanol solution and transported to Molecular biology laboratory at Sokoine University of Agriculture (SUA). Upon arrival, samples were stored at -80ºC until analysis.

3.5 Data Collection Tool

To collect the necessary data, structured questionnaires was developed with reflection of the study objective, the data was collected from 250 households. The questionnaire focused on capturing the knowledge and practices related to ND (Appendix 1).

3.6 Laboratory Analysis

3.6.1 Swabs and tissue preparation

The procedures were carried out in biosafety cabinet class BII where 75 mg of tissue were chopped into smallest pieces and crushed using sterile mortar and pestle.

3.6.2 Viral RNA extraction

The genomic NDV RNA was extracted from the samples using TRIzol™ Reagent (Invitrogen, Carlsbad, CA, USA), following manufacturer instructions. Briefly, the swabs with ethanol (1000 µL) were vortexed for two minutes and 500 µL of supernatant was taken and mixed with 1000 µL of Trizol . On the other hand, crushed tissues with mortar and pestle were mixed with 1000 µL Trizol to solubilize and lyse the cells. Then the mixture was centrifuged at 12 000 × *g* for 5 minutes and the supernatant transferred into new tube, incubated for 5 minutes followed by addition of 200 µL chloroform, the mixture was incubated for 3 minutes and centrifuged for 15 minutes at 12 000 × *g* at 4°C.

The mixture separates into a lower red phenol-chloroform, interphase and a colourless upper aqueous phase. The colourless upper aqueous phase was transferred into new tube and 500 µL of isopropanol added followed by incubation for 10 minutes. There after the mixture was centrifuged for 10 minutes at 12 000 × *g* at 4°C. The supernatant was discarded and the pellet in tube resuspended in 1000 µL of 75% Ethanol, vortexed briefly, then centrifuge for 5 minutes at 7500 × *g* at 4°C. The supernatant was discarded and the pellets air dried for 10 minutes. Finally, the pellets were resuspended in 50 µL of RNase-free water and incubated in water bath set at 60ºC for 15 minutes and stored at -80ºC for PCR assay.

3.7 Conventional One Step Reverse Transcription Polymerase Chain Reaction (RT –PCR)

3.7.1 Preparation of primer stock solution

NDV primers at 100µM stock solution was prepared by adding 880 µL of nuclease free water to the tube containing lyophilized primer. The prepared stock primer was then labeled and stored at -20°C. The working stock of 10 µM concentration was prepared using ratio of 1:10 master stock and nuclease free water. This reduces the number of freeze/thaw cycles that the master primer stock goes through and reduces the chances of contaminating the primary stock of primers.

3.7.2 Amplification of partial fusion gene

The amplification of partial fusion gene was carried out using AgPath-ID™ One-Step RT-PCR (Applied biosystems, Massachusetts, USA), following manufacturer instructions. The NDV target partial fusion gene was amplified using using NDV forward (NDV-F) and reverse primer (NDV-R), (Table 1).

Table 1: Primer names and their sequences

Primer name	Sequences 5' ⟶ 3'	Expected size (bp)
NDV -F	ATGGGCTCCAGACCTTCTACCA	535
NDV-R	CTGCCACTGCTAGTTGTGATAATCC	

3.7.3 Preparation of master mix for PCR

Each of the PCR reaction contained 12.5 µL RT-PCR Buffer, 1 µL NDV-F primer ,1 µL NDV-R primer, 4 µL Nuclease free water, 0.5 µL MgCl, 1 µL RT-PCR Enzymes mix and 5µL of RNA template making a total of 25 µL of reaction mixture. The amplification was carried using master cycler nexus gradient PCR (Eppendorf,

Hamburg, Germany). The PCR conditions were as follows, one cycle of reverse transcription for 30 minutes at 45ºC, one cycle of reverse transcription initial denaturation for 10 minutes at 95ºC,followed by 40 cycles each (consisting of denaturation for 30 sec at 95°C, primer annealing for 30 sec at 55°C and primer extension for 1 minutes at 72°C) and finally one cycle of final extension at 72°C for 7 minutes .The PCR products were then separated on 1.5% agarose gel by agarose gel electrophoresis stained with Gel Red (Phenix, Hong Kong, China). The visualization of the gels containing the separated PCR product were done by using UV Transilluminator.

3.7.4 Sequencing and phylogenetic analysis

PCR products were purified and sequenced using a cycle dideoxy nucleotide sequencer AB13710 (Applied Biosystems, Carlsbad, CA). Nucleotide sequences were edited in sequence scanner software and the reliable consensus sequence of partial fusion gene was generated using bioedit software. The consensus sequence was blasted in NCBI GenBank to determine the homology with other NDV deposited sequences in the NCBI GenBank. The phylogenetic tree was reconstructed by using MEGA X software (Kumar *et al*., 2018).

3.8 Data Analysis

Data from questionnaires were recorded into spreadsheet for statistical analysis. The statistical package for the social sciences (SPSS) computer software was used to generate percentages and the prevalence was calculated by using Microsoft excel. For phylogenetic analyses, the published nucleotide sequences were selected for phylogenetic analysis of MW147368 NDV/Chicken/Tanzania/Iringa/2020 and some

NDV strain lasota and NDV strain I-2 were included. The nucleotide sequence was aligned using Clustal Wallis in MEGA X software. The phylogenetic analysis was done using maximum likelihood method and Kimura 2 parameter model (Kimura, 1980) built in MEGA X software. The phylogenetic tree was generated based on 44 nucleotides sequences of NDV fusion gene which involved 44 nucleotide sequences one being from the field and 43 from the reference strains obtained from NCBI GenBank.

CHAPTER FOUR

4.0 RESULTS

4.1 Demographic Characteristics

Age, sex and residence of respondents is presented in Table 2. The findings reveal that most of the respondents were more than 30 years old and constituted about 83.2% of all the study population. The second group ranged between 25-30 years comprising 14.4% of the study population, while the least group ranged from 18- 24 years old and consisted of 2.4% of all respondents. Most of the interviewed population were male comprising of 77.2% of the study population.

Table 2: Social demographic characteristic of participants (N = 250)

Variable		Frequency	Percentage (%)
Age in years	18- 24	6	2.4
	25-30	36	14.4
	Above 30	208	83.2
Sex	Male	193	77.2
	Female	57	22.8
Residence	Urban	0	0.0
	Rural	250	100

4.2 Knowledge of Chicken Keepers on Newcastle Disease

Table 3 shows that 100% of the participants (farmers) who kept chickens in the backyard were familiar with ND out of which 91.2% experienced ND in their farms. All respondents (100%) were aware that there is vaccine for the ND out of which 69.6% vaccinated their chicken and 30.4 % with no history of vaccinating their chickens. Most respondents (55.2 %) were following a correct vaccination regime by vaccinating three times per year, while 29.6% of respondents were vaccinating only once and 15.2% never vaccinated their chickens.

Table 3: General knowledge of the respondents on Newcastle disease (N= 250 respondents)

Variable		Frequency	Percentage (%)
Awareness with ND	Yes	250	100
	No	0	0.0
Experience of ND infection	Yes	228	91.2
	No	22	8.8
Awareness with presence of vaccine	Yes	241	96.4
	No	9	3.6
Record of vaccinating chickens	Yes	174	69.6
	No	76	30.4
Frequency of vaccinating /year	Never	38	15.2
	Once	74	29.6
	Twice	0	0.0
	Thrice	138	55.2

4.3 Practices of Chicken Keepers Related to Newcastle Disease

Majority of the respondents (68%) kept chickens only in the backyards while 32% of the respondents owned other birds such as ducks, pigeons, quills, geese and Guinea fowl. All farmers in the study population were buying chickens from markets or their neighbours for keeping in their farms. About 60% of the respondents reported that their chickens interacted with wild birds while 40% of the respondents their chickens did not

interact with wild birds. Most of the respondents (98.80%) agreed that their neighbours were vaccinating their chickens while 1.20% said they were not vaccinating their chickens.

Table 4: Practices of chicken keepers related to Newcastle disease

Variable		Frequency	Percentage (%)
Owning other birds apart from chickens	Yes	80	32
	No	170	68
Interaction of chickens with wild birds	Yes	150	60
	No	10	40
Market /neighbours source of restocking chickens	Yes	250	100
	No	0	0.0
Neighbours vaccinating their chickens	Yes	247	98.80
	No	3	1.20

4.4 Detection of Newcastle Disease Virus by RT-PCR

Total of 384 samples were screened for the presence of NDV RNA using One-step RT-PCR where two samples (0.01%) from tissues were found to be positive for NDV. The positive sample showed a specific band of 535 bp in agarose gel electrophoresis (Figure 3).

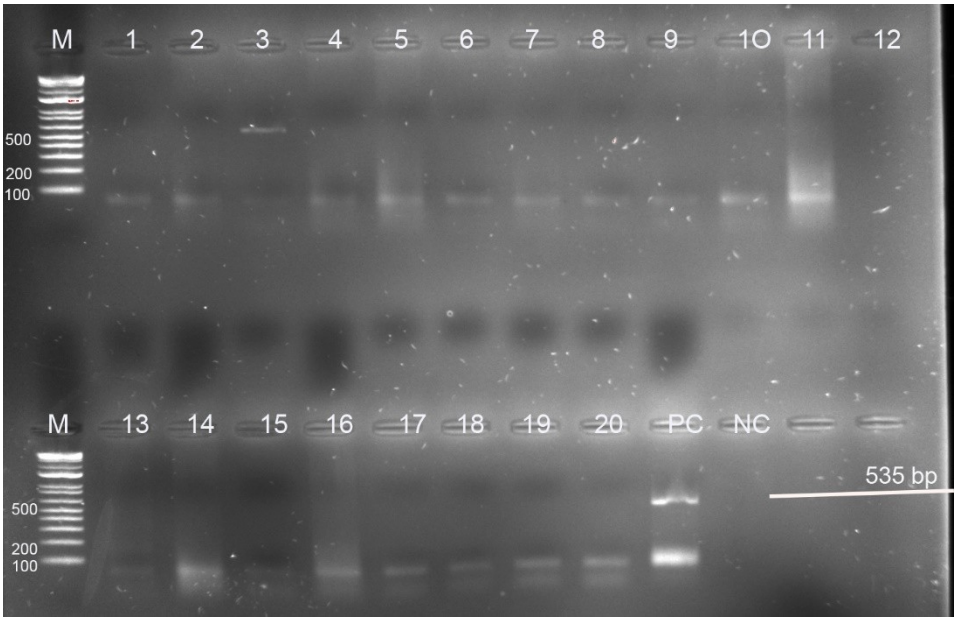

Figure 3: **Gel electrophoresis of the amplified fusion gene of field isolate (MW147368 NDV/Chicken/Tanzania/Iringa/2020).**

The positive sample is 535 base pair (bp) in size, Lane M 100-bp DNA Ladder, Lane 1-20 tested sample identification numbers, Lane PC known positive control sample, Lane NC Negative control (RNase free water)

4.5 Sequence Alignment and Phylogenetic Analysis

A blast analysis showed that MW147368 NDV/Chicken/Tanzania/Iringa/2020 sequence was homology to 100 NDV sequences deposited in the NCBI sequence database. The sequence identity of the 100 sequences ranged from 91 to 98% with highest hit (98%) for Mozambique isolates of 2011 (KX 231366) and 2012 (KU523528) (Table 5). The MW147368 NDV/Chicken/Tanzania/Iringa/2020 isolate clustered together with NDV of genotype seven (G-VII) in the phylogeny but belonged to different genotype with current used vaccine which are Lasota (AY845400) and NDV strain I-2 (AY935499), (Fig. 4).

Table 5: Most closely related NDV from sample collected in the backyard chickens in Iringa rural District

Related virus Acc. number	Nucleotide composition	Nucleotide Match	% Identity	% Differences
KX231366.1	1662	532/535	98	2
KU523528.2	1662	525/535	98	2
MF622041.1	1662	524/535	98	2
KU523529.2	1662	524/535	98	2
KU523526.2	1662	524/535	98	2
KU523524.2	1662	524/535	98	2
MF622043.1	1662	522/535	98	2
MF622037.1	1662	522/535	98	2
MF622036.1	1662	522/535	98	2
KX231367.1	1662	522/535	98	2

GQ901897.1	535	522/535	98	2

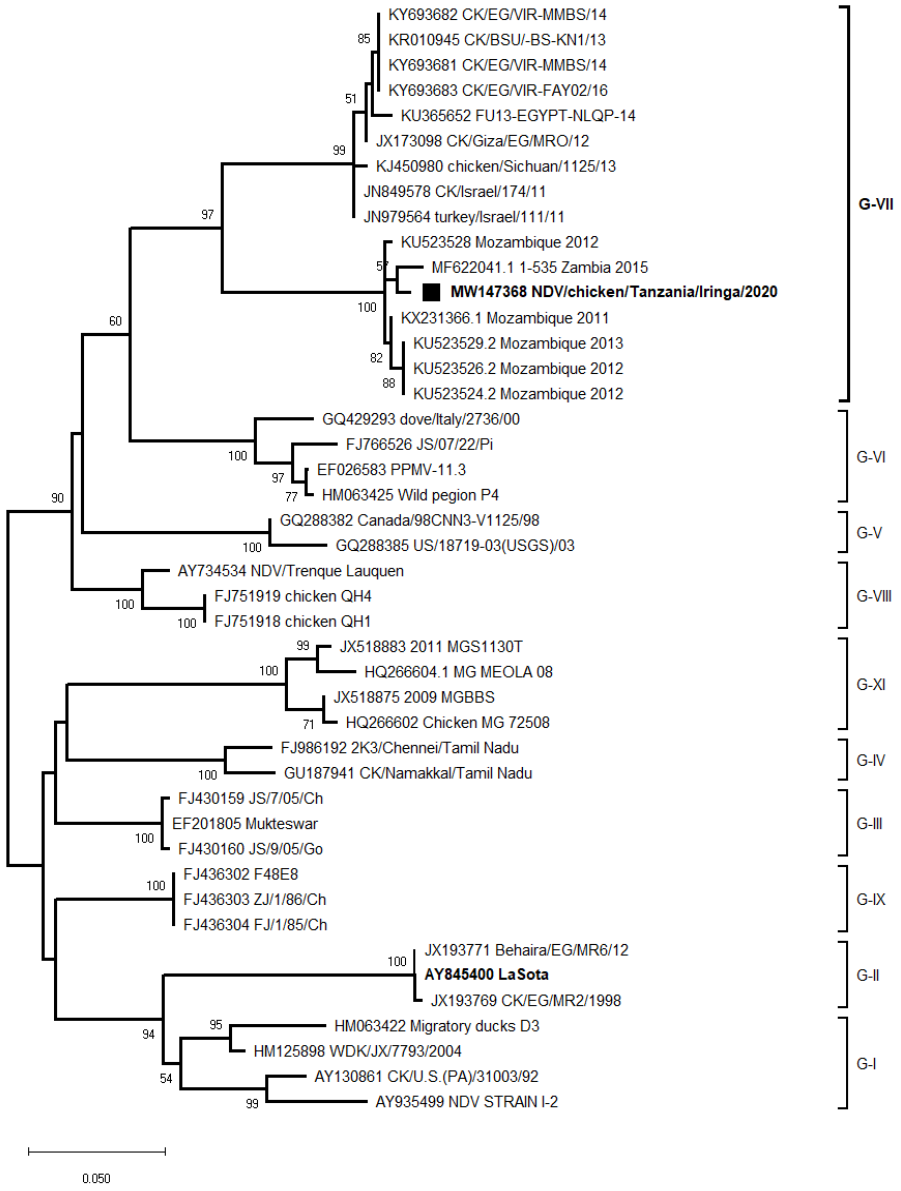

Figure 4: Phylogenetic analysis of NDV from samples collected in the backyard chickens in Iringa rural district. The evolutionary history was inferred by using the Maximum Likelihood and Kimura 2-parameter model (Kimura, 1980) built in MEGA X (Kumar *et al.*, 2018).

CHAPTER FIVE

5.0 DISCUSSION

The study aimed at establishing the epidemiological status of NDV circulating in the backyard chickens in Iringa rural district, focusing on investigating the risk factors for the disease, the prevalence of the disease and genetic characterization of NDV in the study area. With regard to risk factors of ND different questions were subjected to the study population aiming at measuring the population knowledge and practices related to ND. It was observed from the study (Table 3) that 100% of study population were aware with the disease out of which 91.2% experienced the disease in their backyard chickens and 96.4% being familiar that the disease has got vaccine. Regardless the fact that most people were familiar with the presence of vaccine, only 69.6% were vaccinating their chickens with 30.4% having no record of vaccinating their chickens. This could be due to the fact that they are far away from veterinary centers and shops (remoteness) and hence lack of access to vaccine .But also having few numbers of chickens lead to farmers think that it will be more costly to buy the vaccine and follow the proper vaccination regime of the chickens per year which is four times (every three month) for adult chickens and at least six times starting for day one old chicks. The study revealed that 15.2 % had never vaccinated their chickens, 29.6% had vaccinated once and 55.2% had vaccinated at least three times per year. According to the study done at Arusha, Mbeya and Singida by Campbell *et al*. (2018) it was reported that 80% of the household knew that there was a vaccine for ND where by 57% had previously history of vaccinating their chickens and 26% were still vaccinating their chickens following the vaccination regime of the disease. According to the Tanzania Ministry of Agriculture (2012) report it stipulates that only 22% of the poultry farmers regularly vaccinate their

chickens. It can be seen that at least most of the poultry keepers in Iringa do vaccinate their chickens against ND leading to low mortalities due to the disease outbreaks. The knowledge dissemination done by livestock field officer and vaccine supplier companies personnel created awareness on proper vaccination regime and hence reduces mortalities of backyard chickens.

With respect to practices (Table 4) it was determined that 32% of the respondents were owning other birds apart from chickens and 68% owned only chickens, these birds are such as ducks, pigeons, quills, geese and Guinea fowl. Most of these birds are likely to be infected with NDV but they usually not succumb to detrimental effects but act as reservoirs and hence continue maintaining the virus in circulation which also lead to outbreak of disease to chickens especially when the antibody titer goes down. The research done by (Liu *et al*., 2007) on water fowls it was found to be harboring NDV as carrier bird by showing strong resistance to infection but still the isolated virus was threat to chickens and hence this show that the carrier birds play a great role of maintaining the virus circulation. Also, the isolation of the NDV from the healthy ducks revealed the role of ducks in epidemiology of the ND in back yard chickens (Yongolo *et al*., 2002).

With regard to the interaction of chickens with wild birds (Table 4) 60% of the respondent agreed that their chickens are interacting with wild birds while 40% of respondent said there were no interaction. Usually, these wild birds do move from one place to another by doing so they are likely to transmit disease from one place to another as some of them harbor a virulent strain which is fatal to chickens, even if they carry avirulent strains, once is passaged to chicken they become virulent and hence disease outbreak (Shengqing *et al.,* 2002). Gustafson and Moses (1953) demonstrated the

experimental infection of sparrows, suggesting that these birds may transmit the virus to susceptible birds by co-habitation and under natural conditions, sparrows are also infected by NDV and thus can be considered as virus reservoirs for backyard chickens and commercial poultry due to close contacts. Adequate management measures should be adopted in order to avoid the contact of this and other wild species with backyard chickens and commercial poultry for the purpose of reducing the risk of a NDV infection.

The results of present study show that the prevalence of the disease in area is low (0.01%). The samples were collected in March to May 2020 and this was the time after ND outbreak. Normally ND outbreaks occur between September and January. Thus, at the time of sampling most chickens had built immunity reducing the chance of detecting NDV. As previously reported by Kemboi *et al*. (2013) that the reason of failure to isolate virus from swabs and tissue was due to higher Newcastle disease antibody titer which eventually lead to reduced viral titer leading to the difficulty in detecting the virus. This wide range of NDV titer may be due to natural infection which is known to produce higher antibody titers than vaccination (Luc *et al.,* 1992). The continued hatching of chicks and the presence of chickens that survived previous ND outbreaks mean there will always be susceptible chickens which will be infected by the virus (Martin, 1992). The two positive samples for NDV RNA from the study area showed that most of the chickens possibly recovered from the disease and those positive cases could be due to the existing carrier chickens. This sporadic incidence indicates the possibility of persistence of the virus in the area. The low level of NDV RNA detected from chickens' samples may be due to neutralization of the virus by protective antibodies (Alexander, 2003).

Partial sequencing and phylogenetic analysis of NDV F-genes is closely related to isolate of Mozambique with accession number KU523528 and KX 231366. Possibly this is due to the chicken's business across Tanzania and Mozambique for the sake of improving the breeds of chickens in such a way the disease can spread in either direction especially when there is unfaithful worker or illegal importation or exportation across the country borders. The study done in Nigeria shown the relatedness of NDV strain with that of Cameroon indicate a potential trans-border virus transmission through trade in live bird (Solomon, 2012) also Msoffe *et al*. (2019) reported on closely relatedness of virus isolated from Mbeya and that isolated from Mozambique due to trade of live birds. Moreover, the isolated strain belongs to different genotype with the currently used vaccines, similarly Salim *et al.* (2018) after phylogenetic analysis revealed the marked genetic distance between the commercially available ND vaccines and Egyptian isolates. The circulating NDV genotype is still threatening the chickens regardless the vaccination. Continuously monitoring of evolutions of NDV together with periodic evaluation of NDV vaccine and applied vaccination strategies is required.

CHAPTER SIX

6.0 CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

The study revealed that ND is circulating in the backyard chickens in Iringa rural district. Therefore, it is still a threat in village backyard chickens and they can act as the source of infection in commercial poultry production. Moreover, the study revealed that many backyards chickens farmers are familiar with the ND and control strategies through vaccination however few are practicing. Analysis of the detected isolates indicates that the NDV belongs to genotype VII. This study provides the essential information on epidemiology of circulating NDV in Iringa thus highlights importance of continuous surveillance of ND in backyard chickens.

6.2 Recommendations

i. There should be intensive education among backyard chicken keepers in Iringa rural district on proper management of chickens and adherence to veterinary advises.
ii. There should be massive vaccination campaign of all backyard chickens.
iii. There should be regular surveillance and molecular characterization of the circulating NDV genotype so as to notice if there is an on -going evolution of the NDV and to see if it matches with the currently used vaccine.

REFERENCES

Alexander, D. J. (1988). *Historical Aspects, in Newcastle Disease.* (Edited by Alexander, D. J.), Kluwer Academic Publishers, Boston. 11pp.

Alexander, D. J. (2000). Newcastle disease and other avian paramyxoviruses. *Revue Scientifique et Technique-Office International des Epizooties* 19(2): 443 – 455.

Alexander, D. J. (2003). Newcastle disease, other paramyxoviridae and pneumovirus infections. In: *Diseases of Poultry*. (Edited by Saif, Y. M., Barnes, H. J., Glossons, G. R., Fadly, M. A. McDougald, D. J. and Swayne, D.E.), Iowa State Press, Ames. pp. 63 – 100.

Awan, M. A., Otte, M. J. and James, A. D. (1994). The epidemiology of Newcastle disease in rural poultry: a review. *Avian Pathology* 23(3): 405 – 423.

Bell, J. G., Kane, M. and Lejan, C. (1990). An investigation of the disease status of village poultry in Mauritania. *Preventive Veterinary Medicine* 8(4): 291 – 294.

Campbell, Z. A., Marsh, T. L., Mpolya, E. A., Thumbi, S. M. and Palmer, G. H. (2018). Newcastle disease vaccine adoption by smallholder households in Tanzania: Identifying determinants and barriers. *PloS One* 10(1371): 1 – 16.

Chen, J. P. and Wang, C. H. (2002). Clinical epidemiologic and experimental evidence for the transmission of newcastle disease virus through eggs. *Avian Diseases* 46: 461 – 464.

De leeuw, O. S., Koch, G., Hartog, L., Ravenshorst, N. and Peeters, B. P. (2005). Virulence of Newcastle disease virus is determined by the cleavage site of the fusion protein and by both the stem region and globular head of the haemaglutinin-neuraminidase protein. *Journal of General Virology* 86: 1759 – 1769.

Ewies, S. S., Ali, A., Tamam, S. M. and Madbouly, H. M. (2017). Molecular characterization of Newcastle disease virus (genotype VII) from broiler chickens in Egypt. *Beni-Suef University Journal of Basic and Applied Sciences* 6(3): 232-237.

Fan, S., Wang, T., Gao, X., Ying, Y., Li, X., Li, Y. and Xu, Y. (2015). Phylogenetic analysis of Newcastle disease viruses isolated from wild birds in the Poyang Lake region of China. *Journal of Veterinary Medical Science* 2015: 14 – 80.

Ganar, K., Das, M., Sinha, S. and Kumar, S. (2014). Newcastle disease virus: current status and our understanding. *Virus Research* 184: 71 – 81.

Gotoh, B., Ohnishi, Y., Inocencio, N. M., Esaki, E., Nakayama, K., Barr, P. J., Thomas, G. and Nagai, Y. (1992). Mammalian subtilism-related proteinases in cleavage activation of the paramyxovirus fusion glycoprotein: superiority of furin/PACE to PC2 or PC1/PC3. *Journal of Virology* 66: 6391 – 6397.

Gustafson, D. P. and Moses, H. E. (1953). The English sparrow as a natural carrier of Newcastle disease virus. *American Journal of Veterinary Research* 701: 581 – 585.

Intervet (1972). *Important Poultry Diseases*. Schering-Plough Animal Health, Bendigo, Australia. 90pp.

Kaleta, E. F. and Baldauf, C. (1988). *Newcastle Disease in Free-Living and Pet Birds.* Institutfur Gefliigelkrankheiten, Deutschland. 2pp.

Kemboi, D. C., Chegeh, H. W., Bebora, L. C., Maingi, N., Nyaga, P. N., Mbuthia, P. G., Njagi, L. W. and Githinji, J. M. (2013). Seasonal Newcastle disease antibody titer dynamics in village chickens of Mbeere District, Eastern Province, Kenya. *Livestock Research for Rural Development* 25(10): 1 – 9.

Kimura M. (1980). A simple method for estimating evolutionary rate of base substitutions through comparative studies of nucleotide sequences. *Journal of Molecular Evolution* 16: 111 – 120.

Kumar, S., Stecher, G., Li, M., Knyaz, C. and Tamura, K. (2018). Mega X: molecular evolutionary genetics analysis across computing platforms. *Molecular Biology and Evolution* 35(6): 1547 – 1549.

Li, X., Chai, T., Wang, Z., Song, C., Cao, H., Liu, J. and Miao, Z. (2009). Occurrence and transmission of Newcastle disease virus aerosol originating from infected chickens under experimental conditions. *Veterinary Microbiology* 136(4): 226 – 232.

Liu, H., Wang, Z., Wu, Y., Zheng, D., Sun, C., Bi, D. and Xu, T. (2007). Molecular epidemiological analysis of Newcastle disease virus isolated in China in 2005. *Journal of Virological Methods* 140(2): 206 – 211.

Liu, X. F., Wan, H. Q., Ni, X. X., Wu, Y. T. and Liu, W. B. (2003). Pathotypical and genotypical characterization of strains of Newcastle disease virus isolated from outbreaks in chicken and goose flocks in some regions of China during 1985-2001. *Archives of Virology* 148: 1387 – 1403.

Luc, P. V., Hong, N. T. and Chinh, V. T. (1992). Levels of anti-Newcastle disease virus antibodies in industrial poultry at various ages and seasons. *Agricultural Food Industry* 9: 348 – 350.

Martin, P. A. J. (1992). The epidemiology of Newcastle disease in village chickens. In: P.B Spadbrow (Ed). In: *Proceeding of the 39th International Workshop on Newcastle Disease in Village Chickens. Control with Thermostable Oral Vaccines, 1991*. Australia Centre for International Agricultural Research, Kuala Lumpar, Malaysia. pp. 40 – 45.

McBride, M. D., Hird, D. W., Carpenter, T. E., Snipes, K. P., Danaye-Elmi, C. and Utterback, W. W. (1991). Health survey of backyard poultry and other avian species located within one mile of commercial California meat-turkey flocks. *Avian Diseases*. pp. 403-407.

Msoffe, P. L., Chiwanga, G. H., Cardona, C. J., Miller, P. J.and Suarez, D. L. (2019). Isolation and Characterization of Newcastle Disease Virus from Live Bird Markets in Tanzania. *Avian Diseases* 63(4): 634-640.

Naing, L., Winn, T. and Rusli, B. N. (2006). Practical Issues in Calculating the sample size for prevalence studies. *Archives of Orofacial Sciences* 1: 9 – 14.

OIE (2009). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals* (*Mammals, Bird and Bees*). (7th Ed.), World Organization for Animal Health, Paris, France. 624pp.

Panda, A., Huang, Z., Elankumaran, S., Rockemann, D. D. and Samal, S. K. (2004). Role of fusion protein cleavage site in the virulence of Newcastle disease virus. *Microbial Pathogenesis* 36: 1-10.

Pedersen, J. C., Senne, D. A., Woolcock, P. R., Kinde, H., King, D. J., Wise, M. G., Panigrahy, B. and Seal, B. S. (2004). Phylogenetic relationships among virulent Newcastle disease virus isolates from the 2002-2003 outbreaks in California and other recent outbreaks in North America. *Journal of Clinical Microbiology* 42: 2329 – 2334.

Peroulis-kourtis, I. K., Riley, O., Condron, D. G. and Ainsworth, C. (2002). Molecular characterization of Victorian Newcastle disease virus isolates from 1976 to 1999. *Australian Veterinary Journal* 80(7): 422 – 424.

Seal, B. S., Wise, M. G., Pedersen, J. C., Senne, D. A., Alvarez, R., Scott, M. S., King, D. J. and Kapczynski, D. R. (2005). Genomic sequences of low virulence avian paramyxovirus-1 (Newcastle disease virus) isolates obtained from live bird markets in North America not related to commonly utilized commercial vaccine strains. *Veterinary Microbiology* 106: 7 – 16.

Selim, K. M., Selim, A., Arafa, A., Hussein, H. A. and Elsanousi, A. A. (2018). Molecular characterization of full fusion protein (F) of Newcastle disease virus genotype VIId isolated from Egypt during 2012-2016. *Veterinary World* 11(7): 930 – 938.

Shengqing, Y., Kishida, N., Ito, H., Kida, H., Otsuki, K., Kawaoka, Y. and Ito, T. (2002). Generation of velogenic Newcastle disease viruses from a nonpathogenic waterfowl isolate by pas-saging in chickens. *Virology* 301: 206 – 211.

Solomon, P. (2012). *Molecular characterization of Newcastle disease viruses from live bird markets in Nigeria.* Dissertation for Award of MSc Degree at University of Pretoria, South Africa. 108pp.

United Republic of Tanzania (2012). *National Sample Census of Agriculture. Livestock Sector.* Ministry of Agriculture, Dar es Salaam, Tanzania. 539pp.

Yongolo, M. G. S., Machangu, A. M. and Minga, U. M. (2002). *Newcastle Disease and Infectious Bursal Disease among Free-Range Village Chickens in Tanzania*. Sokoine University of Agriculture, Morogoro, Tanzania. 10pp.

Yusoff, K. and Wen, S. T. (2001). Newcastle disease virus: macromolecules and opportunities. *Avian Pathology* 30: 439 – 455.

APPENDECIES

Appendix 1: Questionnaire for assessing risk factors of Newcastle disease in Iringa rural district

Date and time of interview ------/------2019/2020-----:------a.m./p.m.

Name of the respondent ----------

Age and sex , Age; ----------years, Sex: Male /female

Village ----------

I. General Information

1. How many chickens do you have? ----------

2. When did you started keeping chickens? ----------

3. Which breed/kind /type of chickens are you keeping? ----------

II. General Description of Disease and Vaccine

1. Are you familiar with any diseases affecting chickens? --------- (1=Yes, 2=No)

   If yes mention them ……..

2. Do you know the disease called Newcastle? ---------- (1=Yes, 2 =No)

   If yes mention the clinical signs ----------

3. Have you ever experienced this problem at your chickens? -------- (1=Yes, 2 =No)

4. Do you know that there is a vaccine for the disease? ---------- (1=Yes, 2 =No)

   If yes: Have you ever vaccinated your chickens? --------- (1=Yes, 2 =No)

   If yes at what age ----------and how many times ----------

   If no what other alternative do you use to protect your chickens from getting the disease? ----------

5. Where are you buying the vaccines? ----------

6. Do you know how the vaccine is stored? -------- (1=Yes, 2 =No)

If yes: How do you store and handle the vaccine after buying? ……

7. Do you know how the chickens are supposed to be given the vaccine? --------

(1=Yes, 2 =No)

If yes explain how ----------

8. Have you ever experienced death of chickens after vaccination? ------ (1=Yes, 2 =No)

III. General description of the risk factors of the disease

1. Do you keep any other birds apart from chickens? ---------- (1=Yes, 2 =No)

If yes mention them ----------

2. Is there any contact of your chickens with wild birds? ---------- (1=Yes, 2 =No)

3. Do you buy the chickens for restocking from markets or neighbours? ----------

(1=Yes, 2 =No)

4. Are your neighbors keeping chickens? ---------- (1=Yes, 2 =No)

If yes; is there any interaction with your chickens? ----------(1=Yes ,2 =No)

5. Are your neighbours vaccinating their chickens? ---------- (1=Yes, 2 =No)