DETECTION OF NEWCASTLE DISEASE VIRUS IN LAUGHING DOVES (*STREPTOPELIA SENEGALENSIS*) AND CHICKENS IN BACKYARD POULTRY FARMS AND THE LIVEBIRD MARKET IN SAMARU, ZARIA, NIGERIA

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DEPARTMENT OF VETERINARY PUBLIC HEALTH AND PREVENTIVE MEDICINE,
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DECLARATION

I declare that the work in this Dissertation entitled "Detection of Newcastle Disease Virus in Laughing Doves (*Streptopelia senegalensis*) and Chickens in Backyard Poultry Farms and the Live Bird Market in Samaru, Zaria, Nigeria"has been carried out by me in the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, Ahmadu Bello University, Zaria. The information derived from the literature has been duly acknowledged in the text and a list of references provided. No part of this dissertation was previously presented for another degree or diploma at this or any other Institution.

Jerome Unubi, OKPANACHI	_______________	________________
Name of Student	Signature	Date

CERTIFICATION

This Dissertation entitled DETECTION OF NEWCASTLE DISEASE VIRUS IN LAUGHING DOVES (*STREPTOPELIA SENEGALENSIS*) AND CHICKENSIN BACKYARD POULTRY FARMS AND THE LIVE BIRD MARKET IN SAMARU, ZARIA, NIGERIA by Jerome Unubi OKPANACHI meets the regulations governing the award of the degree of a MASTER OF SCIENCE IN VETERINARY EPIDEMIOLOGY of the Ahmadu Bello University, and is approved for its contribution to knowledge and literary presentation.

Professor J.U. Umoh Chairman, Supervisory Committee	_________________ Signature	_____________ Date
Dr. G. S. N. Kia Member, Supervisory Committee	_________________ Signature	_____________ Date
Dr. A. Dzikwi Member, Supervisory Committee	_________________ Signature	_____________ Date
Professor E.C. Okolocha Head, Department of Veterinary Public Health and Preventive Medicine Ahmadu Bello University	_________________ Signature	_____________ Date
Professor K. Bala Dean, School of Postgraduate Studies Ahmadu Bello University, Zaria.	_________________ Signature	_____________ Date

DEDICATION

I dedicate this work to God the Father, God the Son and God the Holy Spirit, the Three Divine Persons of the Blessed Trinity in One True God. It is in our Lord Jesus Christ, the Word of God that we live, and move and have our being, without whom we can do nothing. I can surely do all things through Christ who strengthens me. 'How I love your law, meditating on it all day long! Your command which is mine forever has made me wiser than my enemy. I have more insight than my teachers, for I meditate on your decrees. I have more understanding than the elders, for I abide by your precepts. I turn my feet from evil paths, so that I may keep your word. I have not departed from your decrees for you yourself have instructed me.' - Psalm 119:97-102.

I also dedicate this work to my loving, caring, and understanding family.

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ABSTRACT

Newcastle disease (ND) is a highly infectious viral disease of birds caused by Newcastle disease virus (NDV). Doves have been incriminated in previous outbreaks of the disease in chickens in some parts of the world. Newcastle disease is the most important enzootic viral disease of chickens in Zaria with annual outbreaks that discourage backyard poultry production. The objectives of the study were to detect and characterize NDV in laughing doves and backyard chickens, and to assess biosecurity measures and farm management practices in relation to outbreak of ND as recalled by backyard poultry farmers. This cross sectional study (September 2014 to February 2015)was conducted on 184 swabs from cloacae and pharynxes of 67 trapped laughing doves and 25 backyard chickens from residential areas of Samaru-Zaria, Nigeria, by convenience sampling. Haemagglutination assay (HA) followed by haemagglutination inhibition (HI) test were performed using positive oropharyngeal and cloacal swabs as antigen and hyperimmune serum respectively. Red blood cell adsorption-de-absorption concentration of NDV followed by conventional reverse transcriptase polymerase chain reaction (RT-PCR) were conducted on the HA and HI positive samplesfor molecular detection of NDV. Biosecurity assessment of backyard poultry farms was conducted by face-to-face interview of backyard poultry farmers.Pre-tested semi-structured questionnaires and on the farm inspection were used to assess biosecurity measures, owner's reported outbreak ofND, and farm management practices that favour the outbreak of ND in backyard poultry. This study showed that of the 65.7% (88/134) of dove oropharyngeal and cloacal swabs positive by HA, 42.1% (37/88) were HI positive. Of the 37 HI positives, 21(56.8%) were RT-PCR positive of which eight were lentogenic, twelve were velogenic while one had both lentogenic and velogenic NDV.

While of the 50 chicken oropharyngeal and cloacal swabs screened, 46% (23/50) were HA positive, and of these 69.6% (16/23) were positive by HI. Only 25% (4/16) of the HI positives produced bands after RT-PCR and gel electrophoresis indicative of 3 lentogenic and a velogenic NDV. Also, the biosecurity assessment study revealed that sun-drying household grains and or flour, overstocking above 300 birds, the peak of the rains (August) and start of the cold dry season (November) and accessibility of laughing doves to poultry feed were statistically associated ($P<0.05$) with farmers' reported outbreaks of ND. From the study it was concluded that laughing doves were demonstrated to be infected with either lentogenic or velogenic NDV or both. Also, detection of NDV in laughing doves corresponded with detection of NDV in backyard chickens (with statistically greater detection of NDV in chickens and doves in cluster 1 than cluster 2). The use of red blood adsorption-de-adsorption concentration of NDV enhanced RT-PCR detection using fusion gene primers NDV-F 4829 and NDV-R 5031. The detection of not only lentogenic but velogenic NDV in laughing doves poses a great risk to backyard poultry production. An epizootic of velogenic ND in wild birds could lead to an epizootic in backyard poultry with accompanying economic loss. It is recommended that veterinary agencies of Government should encourage more research on the role of laughing doves in the spread of ND to poultry.

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ABBREVIATIONS, DEFINITIONS AND SYMBOLS

%	percent
µl	microlitre
µM	microMolar
^{o}C	degree centigrade
A.B.U.	Ahmadu Bello University
APMV	Avian Paramyxovirus
bp	base pair
cDNA	complementary Deoxyribonucleic Acid
Chi sq	Chi Square statistic
CI	Confidence Interval
C1	Cluster 1 (Areas BZ, C, E, and G)
C2	Cluster 2 (Areas H and Silver Jubilee, and Palladan)
CS	Cloacal Swab
C-terminus	Carboxyl terminus
DGV	Dextrose Gelatin Veronal
EDTA	Ethylenediaminetetraacetic acid

END	Exotic Newcastle Disease
ENDV	Exotic Newcastle Disease Virus
F-gene	Fusion gene
HA	Haemagglutination Assay
HI	Haemagglutination Inhibition assay
IZSVe	Instituto Zooprofilattico Sperimentale delle Venezie
Kb	kilo base
MDT	Mean Death Time
Min	minute
ml	millilitre
mM	milliMolar
ND	Newcastle Disease
NDV	Newcastle Disease Virus
NDVF	Newcastle Disease Virus Forward
NDVR	Newcastle Disease Virus Reverse
N-terminus	Amino terminus
NVRI	National Veterinary Research Institute

OIE	Office International des Epizooties
OPS	Oropharyngeal Swab
OR	Odd Ratio
PBS	Phosphate Buffered Saline
pH	power of hydrogen
PMV	Paramyxovirus
PPMV	Pigeon Paramyxovirus
RBC	Red Blood Cell
RNA	Ribonucleic Acid
rRT-PCR	real-time Reverse Transcriptase – Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase – Polymerase Chain Reaction
SDS	Sodium Dodecyl Sulphate
SLBM	Samaru Live Bird Market
SPSS	Statistical Package for Social Sciences
SYBR	An Asymmetrical cyanine dye used as a nucleic acid stain in molecular biology
Taq	Thermus aquaticus
UAE	United Arab Emirate

VNND	Velogenic Neurotropic Newcastle Disease
VTM	Viral Transport Medium
VVND	Velogenic Viscetropic Newcastle Disease
Z-score	Standard Score

Definition of terms:

- Laughing doves are species of doves known as *Streptopaedia senegalensis*.
- Newcastle Disease virus is a haemagglutinating virus that causes haemagglutination in washed chicken red blood cells and whose haemagglutination is inhibited by hyper immune serum containing Newcastle disease virus specific antibodies.
- Exotic Newcastle Disease Virus (ENDV) is velogenic viscerotropic and or neurotropic Newcastle disease virus with capability of causing high morbidity and mortality in poultry.
- Functioning Germicide footbath is the use of germicide solution in the footbath placed in front of the door to the poultry pen where those entering or leaving the poultry pen have to dip their footwear to disinfect them.
- Intact chicken-wire barricade is the use of unbroken chicken-wire fencing to keep out laughing doves from entering into the poultry pen.
- Specific footwear per pen is the use of farm-boots or slippers allotted per pen only.
- Trees and pylons are tall objects that serve as nesting and perching places for laughing doves.

- Visitors include family friends and neighbours of backyard poultry farmers, and poultry buyers, egg buyers, and manure haulers who patronize backyard poultry farmers.
- Feed spillage is the spilling of poultry feed onto the ground or premises of backyard poultry pens which may serve as attractants for laughing doves perched on trees or pylons close by.
- Sun-drying grains and flour includes the spreading out of grains or flour of cereals, legumes and other crops in the sun to dry that might be attractive to laughing doves as food.
- Good disposal of litter means the bagging up and sealing of spent litter for sale to manure vendors.
- Storing poultry feed indoors means keeping feed in an enclosure where doves can't access.
- Not owning pigeons or doves means no member of the household with backyard poultry keeps domestic pigeons or doves.
- Newcastle disease outbreak means the farmer says he or she has witnessed an outbreak of Newcastle disease in their backyard poultry farms characterized by pathognomic nervous, enteric and or respiratory signs attributable to Newcastle disease.
- Morbidity pattern means the number of birds affected with the predominating systemic signs in an outbreak of Newcastle disease in a respondent's backyard poultry farm.

- Mortality pattern means the number of birds that died from an outbreak of Newcastle disease which gives a hint to the pathotype of the Newcastle disease virus.
- Intervention taken means what action was taken following an outbreak of Newcastle disease in the backyard poultry.
- Newcastle disease vaccination means the use of LaSota vaccine to immunize the poultry flock at least once.
- Other farmers affected means whether the respondent whose flock suffered an outbreak of Newcastle disease knew if neighbouring backyard poultry farmers had a similar outbreak.
- Latest Newcastle disease outbreak means the most recent outbreak of Newcastle disease in a respondent's backyard poultry farm.
- Signs in affected sick birds mean the syndrome of signs shown by the sick poultry in the latest outbreak Newcastle disease in the respondent's flock.
- Lesions in posted dead birds mean the predominant lesions in a dead or moribund chicken in the latest outbreak of Newcastle disease in a respondent's flock that was posted by a Veterinary doctor or certified veterinary attendant.
- Sudden death means the death of poultry overnight or within a short time without premonitory signs in a backyard poultry flock affected by an outbreak of Newcastle disease.
- All-in-all-out policy means the stocking and disposal of poultry flock as a whole batch with no new additions or replacements of dead ones during the grow-out and production periods respectively for broilers and layers.

- Good sanitation and hygiene means the premises of the poultry house are swept daily or weekly to prevent buildup of spilled feed and food particles around the pen thereby reducing the chances of laughing doves loitering/foraging around the pen.
- Hired hands means poultry workers who are hired by backyard poultry farmers.
- Effective disposal of waste excess feed means proper evacuation of stale or feed spilled on the ground into closed bins to prevent attracting laughing doves.
- Effective disposal of mortalities means disposing dead birds by incineration or deep burial.
- Owning a farm record book means the keeping and daily updating of events and procedures on the poultry farm.

CHAPTER ONE

INTRODUCTION

1.1 Background Information

Velogenic Newcastle disease (ND) also known as exotic Newcastle disease (END) is a contagious and fatal viral disease affecting all species of birds. Exotic Newcastle disease is one of the most infectious diseases of poultry in the world and is so deadly that birds die without showing any signs of disease. A mortality rate of almost a hundred percent can occur in unvaccinated poultry flocks. Velogenic Newcastle diseasevirus can infect and cause death even in vaccinated birds (Margaret, 2006). Exotic Newcastle disease affects the respiratory, nervous and digestive systems of birds. The incubation period of Newcastle disease ranges from 2-15 days. Signs include sneezing, gasping for air, nasal discharge, coughing; greenish watery diarrhoea; depression, muscular tremors, drooping wings, twisting of the head and neck (torticollis), circling, complete paralysis; partial to complete drop in egg production, production of thin-shelled eggs; swelling of tissues around the eyes and in the neck; sudden death and increased deaths in a flock (USDA website, 2014). Exotic Newcastle disease is spread by direct contact between healthy birds and the bodily discharges of infected birds (droppings and nasal, oral or ocular secretions). Exotic Newcastle disease spreads rapidly among birds kept in confinement such as commercially raised chickens. Shoes, clothing and equipment of vaccination and debeaking crews, manure haulers, truck drivers, feed delivery personnel, poultry buyers, egg service people and poultry farm owners and employees all serve as agents of spread of exotic Newcastle disease virus (Margaret, 2006). Prevention of Newcastle disease is by ensuring optimum bio-security standards for poultry farms. All dead wild birds in a farm should be handled as Trojan horses containing an unknown number of pathogenic agents, so treated as suspected contagion (which should be picked up as soon as they are found with gloved hands, placed in a plastic bag, sealed, sent to a reference virology laboratory for diagnosis and feedback report) (USDA website, 2014).

The "gold standard" for identification of Newcastle disease virus is isolation and cultivation in embryonated chicken eggs followed by haemagglutination test (HA), haemagglutination inhibition test (HI) and pathotyping of the virus. Pathogenicity traditionally is determined by the intracerebral pathogenicity index. These assays are labour intensive and time-consuming, requiring up to ten days in completing and avoidable suffering to embryos and destruction of animal life. This hinders the authorities in undertaking adequate measures in a timely manner to limit the spread and eradicate the infection. The development and implementation of rapid, reliable, and high-throughput diagnostic methods for detection of the virus could provide a valuable contribution to controlling the disease. The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) is a test that satisfies the requirements for high sensitivity and specificity coupled with a short turnaround time (Dimitrov *et al.*, 2014). The F gene of virulent NDV (END) has a unique pair of dibasic amino acids at the cleavage site (115 and 116 with a phenylalanine at 117) and a basic amino acid at 113 while lentogenic strains have monobasic amino acids and lack dibasic amino acids (Naresh *et al.*, 2009). Bruce *et al.* (1995) using Reverse Transcription Polymerase Chain Reaction (RT-PCR) coupled with Direct Nucleotide Sequencing and Sequence Database for NDV pathotype prediction, showed that lentogenic vaccine isolates LaSota, England/F, B1, and Queensland/V4 and the lentogenic field isolate 91/33 all have the sequence 109-SGGGRQGRLIG-119 at the Fusion Cleavage site, while the mesogenic and velogenic viruses have the sequence 109-SGGRRQR(K)RFIG-119 containing the two diagnostic pairs of dibasic amino acids associated with virulence. Furthermore, virulent isolates have the RR sequence instead of the GR sequence at positions 112 and 113 as well as RR or RK pair rather than the GR sequence at positions 115 and 116. Real Time RT-PCR however is quite expensive for routine diagnostic screening of backyard poultry flocks. Using haemagglutination test (HA), haemagglutination inhibition (HI) and conventional reverse transcription-polymerase chain reaction (RT-PCR) assays would be affordable, reliable and give similar results to real time RT-PCR, enabling effective prevention and control by the veterinary professionals (Bruce *et al*., 1995).

1.2 Statement of Research Problem

Backyard poultry production has come to stay in Nigeria. In Zaria, many residential areas have residents who raise broilers and layers to meet the local demand for chicken meat and eggs (personal communication with respondents). The manure derived as a by-product is often bagged and sold to manure haulers and hawkers for additional revenue generation. This is a profitable business and is mostly managed by the housewives especially in the staff quarters of the Ahmadu Bello University Zaria and other residential areas in Zaria (personal communication with respondents). There is ready and easy access to avian veterinarians and vaccines and poultry feed can readily be obtained in Zaria as well. Most poultry farmers have a vaccination schedule and sometimes employ the services of professional veterinarians or veterinary technicians/attendants in drafting one and carrying out vaccinations. In spite of these prophylactic vaccinations against endemic poultry diseases such as Newcastle disease, Marek's disease and fowl pox, farmers still suffer outbreaks of Newcastle disease that sometimes cripples production to zero (personal communication with respondents). This may be attributed to various reasons which may not be limited to source of birds, transportation, biosecurity measures in the farm, vaccine break and vaccine failure, incursion of poultry houses with wild birds like doves, pigeons, and finches. Oladele *et al.*(1996) and Sa'idu *et al.* (2004) established through serological techniques (HA and HI) that pigeons and doves in Zaria were infected with Newcastle disease virus, just as Wambura (2010) detected antibodies to Newcastle disease virus in guinea fowls and pigeons in Tanzania.

Zaria is within the guinea savanna zone of Nigeria, and there are established populations of doves which inhabit the tall trees present in the Ahmadu Bello University Staff quarters and other residential areas in Zaria with tall trees (personal communication). These doves in foraging for food, visit households to pick up grains, flour, chaff, and chicken feed spilled on the ground around backyard poultry houses, especially when feeding their nestlings (squabs). This increases the chances of dove droppings contaminating the premises of households with backyard poultry. The soles of footwear of poultry farmers may also be contaminated with these wild bird droppings and be transferred into the backyard poultry houses. With broken chicken wire fencing of poultry houses, doves may actually invade such pens and contaminate poultry feed and water with oro-nasal discharges and faeces.

1.3 Justification of the Study

Exotic Newcastle disease, previously known as velogenic viscerotropic Newcastle disease, is so virulent that many birds die before showing any clinical signs (Margaret, 2006). A death rate of one hundred percent can occur in exposed flocks (Margaret, 2006). An END outbreak can jeopardize a state's poultry production and limit its international trading opportunities (Margaret, 2006). Backyard poultry farming contributes to the financial income of families that run them, and ensures that broilers and eggs are readily available to the residents of the A.B.U. staff quarters and environs (personal communication).

This study sought to go a step beyond serological evidence of NDV from previous studies done in Zaria (Oladele et al., 1996), to the detection (HA and HI tests), pathotyping (RT-PCR) and electrophoretyping (agarose gel electrophoreses) of NDV isolated from doves in Zaria, and compared with NDV in chickens. This would prove for certain whether pigeon NDV plays an epidemiological role in the outbreak of ND in chickens.

Using the three diagnostic techniques HA, HI, RT-PCR and visualizing via gel electrophoresis to detect, pathotype and classify NDV over the serological HA/HI, viral isolation via embryonated eggs, MDT, and other laborious time consuming procedures is justified if the results are similar. NDV spreads rapidly, and cutting down the number of diagnostic procedures is of the greatest importance to the economy, the poultry sector and the health of wild birds.

The definition of the Office International des Epizooties (2001) reflects the current understanding of the molecular basis for virulence: Newcastle disease is defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV–1) that meets one of the following criteria for virulence: (a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (*Gallus gallus*) of 0.7 or greater. Or (b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 to 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterisation of the isolated virus by an ICPI test (Aldous and Alexander, 2001).

1.4 Aim of the Study

The aim of the study was to detect and characterize the strains of Newcastle disease viruses (NDV) in laughing doves in Zaria and assess their potential to cause exotic Newcastle disease in domestic poultry.

1.5 Objectives of the Study

The objectives of the study were to:

a. determine the prevalence and pathotype of NDV in laughing doves trapped near backyard poultry farms in Zaria.

b. assess biosecurity measures of backyard poultry farms against laughing doves that prevents the spread of NDV to backyard poultry in Zaria.

c. assess the outbreak of Newcastle disease in chickens of backyard poultry farmers in Zaria.

d. assess backyard poultry farm management practices that affect the spread of NDV between backyard poultry and doves.

1.6 Research Questions

a. Do laughing-doves in Samaru-Zaria harbour Newcastle disease virus (NDV) and are the NDV isolates from laughing-doves velogenic, mesogenic or lentogenic?

b. Does the use of biosecurity measures prevent the spread of ENDV from laughing doves to backyard poultry?

c. Does the history of ND outbreak and signs observed in affected sick chickens of backyard poultry farmers indicate exotic Newcastle disease (END)?

d. Does farm management practice affect the outbreak of END in backyard poultry?

CHAPTER TWO

LITERATURE REVIEW

2.1 Aetiology of Newcastle Disease

Newcastle disease is caused by a paramyxovirus, called PMV-1, one of nine serotypes of this virus identified. There are four large groups of paramyxoviruses based on how dangerous they are and the type of disease that they cause in chickens. PMV-1 can infect a broad range of animals, including many species of mammals (including humans) and most species of birds (Margaret, 2006). Newcastle disease virus formally called avian paramyxovirus-1(APMV-1) causes severe losses in domestic poultry production. It is an enveloped virus with a negatively sensed single-stranded RNA genome of about 15 kilo bases (15kb) which code for 6 proteins, including RNA-directed RNA polymerase (L), haemagglutinin-neuraminidase protein (HN), fusion protein (F), matrix protein (M), phosphoprotein (P) and nucleoprotein (N) (Mark *et al*., 2004).

Two strains of NDV are common in domestic fowl in the United States of America, called lentogenic and mesogenic NDV. There are two foreign strains, called velogenic strains that do not naturally occur in the United States of America (unlike in Nigeria), velogenic viscerotropic Newcastle disease virus (VVNDV) and velogenic neurotropic Newcastle disease virus (VNNDV). To prevent the introduction of VVND and VNND, as well as avian influenza, the United States Department of Agriculture (USDA) restricted the importation of birds in the early 1970's, and set up quarantine stations to monitor imported birds for these viruses. Birds being imported to the United States must be quarantined in a USDA-controlled facility where they will be monitored and tested for PMV-1. Most recently, VVND and VNND have been classified as exotic Newcastle disease (END) (Margaret, 2006).

Velogenic viscerotropic Newcastle disease virus (VVNDV) is the most virulent form of Newcastle disease virus affecting poultry. Smuggling of birds is considered the only route by which VVND virus could enter the United States. However, once it enters, it can spread to any susceptible bird. Poultry that are infected usually die rather quickly. Among exposed poultry, a very high majority will succumb to the virus and die. This VVNDV infection could have very serious repercussions for commercial poultry facilities, as they may sustain extremely high losses among their birds. The incubation period of VVND in birds can vary from 3 to 28 days, depending on the strain, the quantity of virus and susceptibility of the host bird. In psittacine birds, the incubation period ranges from 5 to 16 days. In chickens, the incubation period averages about 5 days (Margaret, 2006).

2.2 Transmission of Newcastle Disease

The disease is transmitted when NDV is shed from an infected bird (in all secretions, but primarily in respiratory secretions) and a susceptible bird either ingests or inhales virus particles. Aerosolized faecal dust from infected birds and contaminated bedding are considered potential sources for infection. Newcastle disease virus is very stable outside of an infected bird, so insects, rodents and humans can disseminate the virus to other susceptible birds. The virus has also been demonstrated to be transmitted from chicken to chicken by feather mites. Free-ranging wild birds should be of minimal importance in spreading the virus and migratory birds appear to have no impact on the spread of the virus. Infected birds may appear normal and shed the virus in their faeces. Amazon parrots may shed the virus for more than a year without showing clinical signs themselves (Margaret, 2006).

Exotic Newcastle disease should not be a concern for professional psittacine breeders and pet owners who avoid birds that have entered the United States illegally. Fighting cocks and other birds illegally smuggled into the United States of America are usually responsible for outbreaks in the USA (Margaret, 2006).

More recently (2006), END had been diagnosed in two wild pigeons in Riverside, California Arizona (CA), which were scavenging feed on a poultry operation, and were found alongside chickens inside coops. Wild pigeons cannot be easily contained and isolated to prevent the spread of the disease (Margaret, 2006).

2.3 Prevention, Control and Decontamination in an Outbreak of Newcastle Disease

Drastic preventative measures are the best defence against END. These include quarantining property where birds have been infected, euthanizing all birds that may have been exposed, and sanitizing the infection site.

Since NDV is stable in the environment, the virus is resistant to many common disinfectants (such as vinegar). The virus has been found to remain active in moist soil for 22 days, on feathers at 20°C for 123 days and in lake water for 19 days. The virus can be inactivated by extremes in pH (less than 2, greater than 11), high temperatures (56°C), sunlight, detergents, chloramines (1%), bleach, phenols and 2% formalin (Margaret, 2006).

2.4 Zoonotic Potential of Newcastle Disease Virus

Avian paramyxovirus-1 can cause disease in humans. Healthy people who are exposed may develop mild signs of infection (malaise) or conjunctivitis. Infected people will shed the virus in secretions for a period of time after exposure, and they will be technically able to pass the virus to other humans or birds, but this is unlikely, as practicing good hygiene should prevent this. The risk of human disease is confined primarily to poultry workers; however any human exposed could develop signs of infection (Margaret, 2006).

2.5 Vaccination against Newcastle Disease

Commercial poultry facilities in the USA, just like in Nigeria, vaccinate their birds against lentogenic (LaSota NDV Vaccine) and mesogenic (Komarov NDV vaccine) NDV. However, they are not vaccinated against exotic Newcastle disease (VVND and VNND) as a policy. Prevention of these diseases is accomplished by strict control of birds imported into the country. Psittacine birds (parrots) and Columbid birds (pigeons and doves) are not vaccinated against lentogenic and mesogenic NDV, as some vaccines contain modified live virus and could possibly cause the disease. This is because the vaccine was not developed for Psittacine or Columbid birds. In general, modified live vaccines and killed virus vaccines should not cause disease they were developed to protect. Since END is a reportable and notifiable disease, government regulations may restrict vaccination of avian species other than commercial fowl. Vaccination against VVND and VNND is performed in some other countries (Margaret, 2006).

2.6 Diagnosis of Newcastle Disease Virus

2.6.1 Conventional diagnosis of Newcastle disease

PMV-1 can be detected in specimens from both live and dead birds. Most commonly, in live birds, swabs of the pharyngeal area and/or cloacae (or faeces) may be tested at appropriate diagnostic laboratories, using virus isolation. These swabs are then tested for the presence of virus by attempting to grow PMV-1. The faeces may also be examined under an electron microscope to look for PMV-1 virus particles. Blood may be drawn to test for an increase in antibody titre (which requires paired serum samples drawn two weeks apart). These serological tests are less effective than virus isolation in diagnosing infections. In live birds, swabs of the pharynx, cloacae or faeces, to attempt virus isolation, are the tests used to screen birds in quarantine and those suspected of being infected. Post-mortem samples for virus isolation should include trachea, lungs, spleen, liver and brain (Margaret, 2006). Based on severity of ND in chickens lentogenic NDV strains cause subclinical infection with mild respiratory or enteric disease and accompanied with low mortality and virulence; mesogenic NDV strains are of intermediate virulence causing respiratory infection with moderate mortality (<10%); while velogenic NDV strains are highly virulent causing mortality rates of up to 100% (Jos *et al.*, 2011). Velogenic Viscerotropic Newcastle disease virus (VVNDV) strains produce lethal haemorrhagic lesions in the viscera, whereas Velogenic Neurotropic Newcastle disease virus (VNNDV) strains cause severe neurological and respiratory disorders in chickens (Jos *et al.*, 2011). Additionally, virulent NDV can replicate in most avian and mammalian cell types without the addition of trypsin while lentogenic NDV require trypsin for replication especially in avian fibroblasts or mammalian cell types (Bruce *et al.*, 1995).

The "gold standard" for identification of NDV involves isolation and cultivation in embryonated chicken eggs followed by haemagglutination test, haemagglutination inhibition test and pathotyping of the virus. Pathogenicity traditionally is determined by the intracerebral pathogenicity index. These assays are labour intensive and time-consuming, requiring up to 10 days completing. The 'gold standard'hinders the authorities in undertaking adequate measures in a timely manner to limit the spread and eradicate the infection. The development and implementation of rapid, reliable, and high-throughput diagnostic methods for detection of the virus could provide a valuable contribution to controlling the disease.

2.6.2 Molecular diagnosis of Newcastle disease.

The real-time reverse transcriptase-polymerase chain reaction (rRT-PCR) test satisfies the requirements for high sensitivity and specificity coupled with a short turnaround time (Dimitrov *et al.*, 2014). The F gene of virulent NDV (END) has a unique pair of dibasic amino acids at the cleavage site (115 and 116 with a phenylalanine at 117) and a basic amino acid at 113 while lentogenic strains have monobasic amino acids and lack dibasic amino acids (Naresh *et al.*, 2009). Bruce *et al.* (1995) using reverse transcription polymerase chain reaction (RT-PCR) coupled with direct nucleotide sequencing and sequence database for NDV pathotype prediction, showed that lentogenic vaccine isolates LaSota, England/F, B1, and Queensland/V4 and the lentogenic field isolate 91/33 all have the sequence 109-SGGGRQGRLIG-119 at the fusion cleavage site, while the mesogenic and velogenic viruses have the sequence 109-SGGRRQR(K)RFIG-119 containing the two diagnostic pairs of dibasic amino acids associated with virulence. Furthermore, more virulent isolates have the RR sequence instead of the GR sequence at positions 112 and 113 as well as RR or RK pair rather than the GR sequence at positions 115 and 116. The following are oligonucleotide primer probes used for positive control, lentogenic and velogenic strains of NDV. Invitro-transcribed RNA for positive control and determination of the limit of detection for the RT-PCR F-gene assay for game chicken/US(CA)/02 cDNA was amplified with the F581R primer 5'-CTG CCA CTG CTA GTT GIG ATA ATC C- 3' at positions 5054 to 5078 bp in the full length NDV genome (Mark *et al.*, 2004). The F+4839 primer (5'-TCC GGA GGA TAC AAG GGT CT-3'), F+4894(VFP-1) primer (5'-[FAM] AAG CGT TTC TGT CTC CTT CCT CCA[TAMRA]-3') (where FAM means 6-carboxyfluoresceine and TAMRA means 6-carboxytetramethylrhodamine) and F-4939 primer (5'-AGC TGT TGC AAC CCC AAG-3') were used to detect velogens. The F+4839 primer-probe set was designed to specifically detect END outbreak in the south-western US and closely related isolates for the probe was able to detect a wide range of velogens and mesogens (Mark *et al.*, 2004). Farkas *et al.* (2009) used the following oligonucleotide primers/probes for rRT-PCR detection of lentogenic and velogenic/mesogenic NDV F gene sequences:

Lentogens: FT_NDV_LF3 primer (5'-TCC GBA GGA TAC AAG AGT CYG TGA CC-3' at position 4839-4864 bp); FT_NDV_LF4 primer (5'-TCC GBA GGA TAC AAG AGT CYG TGA CT-3' amplicon size of 85 bp); FT_NDV_LR2 primer (5'-AGA GCY ACA CCG CCA ATA AT-3' at position 4923-4904 bp); FT_NDV_LR3 primer (5'-AGA GCY

ACA CCA CCG ATA AT-3') and FT_NDV_Lprobe2 primer (5'-CAG GGR CGC CTT ATA-3' at position 4883-4897 bp).

Velogens/mesogens: FT_NDV_VF1 primer (5'-GAY TCY ATC CGY AGG ATA CAA GRG TC-3' at position 4832-4857 bp); FT_NDV_VR2 primer (5'-AAC CCC AAG AGC TAC ACY RCC-3' at position 4930-4910 bp); FT_NDV_VR3 primer (5'-GAC CCC AAG AGC TAC ACY RCC-3' at position 4930-4910 bp); FT_NDV_Vprobe1 primer (5'AAR CGT YTC TGY CTC C-3' at position 4893-4878 bp) and FT_NDV_Vprobe2 primer (5'AGA RAC GTC TTR TAG GTG C-3' at position 4884-4902 bp) where Y=T or C, R=G or A, B=C,G or T, and nucleotide positions correspond to the NDV B1 complete genome (GenBank accession number AF309418).

Mark *et al*. (2004) in an attempt to develop real time Reverse Transcription Polymerase Chain Reaction (rRT-PCR) for detection of NDV RNA from clinical samples, were able to demonstrate by experimentally infecting chickens, that rRT-PCR for the Fusion gene and APMV-1 gene assay at 2 days post infection only 3 of 17 (17.6%) cloacal swabs and 8 of 17 (47.1%) oral swab samples positive by virus isolation were also detected with the F-gene assay and 14 of 17 (82.4%) were positive with the APMV-1 M gene assay. While by 4 days post infection, 88.2% of the cloacal swabs and 89.5% of the oral swabs that were eventually positive by virus isolation were also positive with F gene and APMV-1 M gene assay. In line with studies such as these, the Office International des Epizooties (OIE, 2000) now accepts reporting of the F cleavage sequence as a primary virulence determinant for the NDV pathotypes.

2.6.3 Epidemiological significance of molecular diagnosis of ND.

Since wild birds are considered natural reservoirs of NDV most of which are lentogenic, and may mutate to mesogenic and velogenic forms in domestic poultry, Naresh *et al.* (2009) recommended that epidemiological surveillance studies should be continued to determine the prevalence of lentogenic NDV in wild birds. They also suggested that there was an epidemiological link between isolates recovered from outbreaks of ND in domestic poultry with those obtained from wild bird populations (Naresh *et al.*, 2009).

Chantal *et al*. (2013) revealed that there was a high diversity of genotypes and sub genotypes of NDV in West and Central Africa. In Nigeria, the isolates characterized from 2006 to 2011 belonged to sub genotypes XIVa, XIVb, XVIIa, XVIIb and XVIIIb. Virulent strains exhibited the consensus sequence 112-(R/K)RQ(R/K)R*F-117 (where the asterisk '*' represents the site of cleavage of the precursor protein F0 into its F1 and F2 subunits), at the cleavage site of the F0 precursor, while avirulent strains had the sequence 112-(G/E)(K/R)Q(G/E)R*L-117. This is in line with the OIE's definition with Phenylalanine (F) at residue 117 of the fusion gene sequence for virulent NDV strains and lysine or arginine (L or R) at residue 117 of the fusion gene sequence for avirulent strains of NDV (Aldous and Alexander, 2001).

Owing to the high cost of real-time primers, an epidemiological survey such as this research work on detection of NDV in laughing doves and chickens in backyard poultry and the Samaru live bird market necessitates the use of conventional RT-PCR for the detection of Newcastle disease virus. The following protocol had been used successfully:

Detection and Typing of APMV-1 by End-Point RT-PCR and Restriction Endonuclease Analysis *Protocol 1*.

This one-step RT-PCR protocol has been adapted at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) and was based on the primer set describedby Creelan *et al*. (2002). In this protocol, amplification of APMV-1-specific nucleic acid fragments followedby enzymatic digestion (restriction endonuclease analysis, REA) using *Bgl*I was carried out todetect and type strains according to their virulence.

Compared to virus isolation, the relative sensitivity of this protocol for clinical specimenswas 73.44% on a sample basis, rising to up to 91.30% on a case basis (Creelan *et al*., 2002).Due to the small amplicon size, it was recommended that the RT-PCR results be visualized on silver-stainedSDS-polyacrylamide gels or 2–3% agarose gels.

2.6.4 Modified conventional RT-PCR using RBC adsorption and de-adsorption technique for diagnosis of Newcastle disease virus antigen

Jianzhong and Chengqian (2011), reported that RT-PCR has limited sensitivity when faecal samples and other samples with high contaminants are used giving false results; while combining RT-PCR with chicken red blood cell adsorption was much more sensitive than conventional RT-PCR alone, as the former effectively concentrated and purified the NDV during the extraction process of RT-PCR. Jianzhong and Chengqian (2011) concluded that red blood cell adsorption combined with RT-PCR was useful when the amount of NDV particles were too low to be detected at the onset of an outbreak or when it was necessary to trace the origin of the different viruses causing an outbreak.

2.7 Historical Overview of Newcastle Disease in Doves

Several published studies on ND in pigeons have been carried out in Italy, India, Russia and the United States of America. In Italy, Buonavoglia *et al*. (1991) reported that characterization studies on three strains of NDV, two strains isolated from pigeons and one strain from chickens, revealed that each of the three NDV strains consisted of different clones of genetically mixed viral populations. Their study further revealed that the pigeon NDV isolates were lentogenic based on mean death time (MDT) determination and velogenic based on intracerebral pathogenicity index. Following a 1982 outbreak of ND in Italy, the antigenic and pathogenetic relationship between pigeon NDV isolates and reference pathogen and non-pathogen NDV-strains were investigated. The pigeon isolates were lentogenic by MDT and between lentogenic and mesogenic as measured by Hanson test. The isolates did not produce plaques in chicken embryo fibroblasts and showed high pathogenicity for experimentally infected pigeons, low-pathogenicity for quails and were not virulent for chickens. They were different antigenically from the LaSota strain as measured by the cross-haemagglutination inhibition test and induced considerable seroconversion in inoculated animals. A lentogenic neurotropic pigeon-pathogenic strain was considered (Biancifiori and Fioroni, 1983).

In India, velogenic Newcastle disease virus from racing pigeons were isolated, based on MDT in 10-day-old embryonated chicken's eggs and intravenous pathogenicity index in 6-week-old chickens. This was an unusual virus because it could not be grouped with the available panel of monoclonal antibodies at the World Reference Laboratory for Newcastle Disease, United Kingdom. However commercially available lentogenic and mesogenic vaccines provided full protection to chickens against this unusual pigeon NDV (Roy *et al*., 2000).

In Russia, pigeons in Moscow were plagued by a combination of ND and Salmonellosis, with pigeons falling from the skies disoriented, spiralling, and lethargic. This outbreak was linked to above-average environmental temperatures due to global warming during the mating season of pigeons (Morgana, 2013).

In the 2002-2003 California outbreak of END in backyard fowl and commercial poultry necessitated the destruction of about 3.3 million birds, estimated at 200 million dollars (Mark *et al.*, 2004).

Prior to 1971 no case of natural infection with NDV was reported in pigeons in Europe (Vindevogel and Duchatel, 1988). However, an outbreak of epidemic proportions decimated aviculture in Europe between1971-1973(Vindevogel and Duchatel, 1988). Pigeon paramyxovirus-1(PPMV-1) strains caused outbreaks of ND among racing and show pigeons in Europe in 1981 and re-emerged in 1985 causing a panzootic (Biancifiori and Fioroni, 1983). In 1984, there were 20 reported cases of ND in unvaccinated chickens due to consumption of feed contaminated with infected pigeon faeces (Alexander *et al.*, 1984). In Scotland there was an outbreak of ND in chickens due to NDV of pigeon origin in 2006 (Dilaveris *et al.*, 2007). In pigeons and doves clinical signs of NDV infection include neurological signs such as torticollis and paralysis, and the excretion of large volumes of green, watery diarrhoea (Alexander *et al.*, 1984). By intracerebral pathogenicity (ICPI) values for PPMV-1 are typical of mesogenic NDV though most PPMV-1 isolates increased in virulence for chickens after passage and therefore constitute a valid threat to backyard poultry production (Kommers *et al.*, 2001). PPMV-1 viruses have been isolated from not only pigeons, doves and chickens, but also from kestrels, falcons, cockatoos, budgerigars, pheasants, swans and robins (Alexander, 1998; Aldous *et al.*, 2001).

In Africa, there have been occurrences of ND in chickens and serological studies carried out in wild free range birds and other domestic and semi-domestic birds including pigeon and doves for NDV. There has been serological evidence of ND in pigeons in Zaria (Oladele *et al.*, 1996; Sa'idu *et al.*, 2004). Large die-offs in doves and pigeons have occasionally been reported in various parts of South Africa since the 1980s after the first isolation of PPMV-1 from doves during an outbreak in September 1986 (Pienaar and Cilliers, 1987).

A high percentage of domestic pigeons and doves were positive for antibodies to NDVin Zaria (Sa'idu *et al.*, 2004). This finding may be explained by the fact that domestic pigeons and doves are more closely associated with human dwellings. Although the turtle dove is not domesticated it does, however, lay and hatch its eggs in nests constructed in the eaves of roofs or on branches of trees located in or around human settlements. Thus, because of this close affinity for human habitation, villagers have developed the habit of placing broken pots and similar discarded items on free tops or eaves of roofs to serve as nests for the turtle dove in particular, and to a lesser extent for the laughing dove(Sa'idu *et al.*, 2004). The laughing dove (*Streptopelia senegalensis*) is not scared of humans as much as the turtle dove, and usually gathers around houses where the birds water themselves from spilled water and feed from grains and food material spilled on the ground(Sa'idu *et al.*, 2004). The mourning dove rarely visits or nests around human habitations(Sa'idu *et al.*, 2004). Despite the low HI titres observed in pigeons and in some species of doves in their study, they could serve as a source of infection for both local and exotic chickens (Sa'idu *et al.*, 2004). This observation has been demonstrated by the 20 ND outbreaks in Britain that were reported in unvaccinated chickens as a result of consumption of feed that was contaminated by droppings from infected but clinically normal pigeons (Alexander *et al*., 1984). The laughing dove is a common and widespread species in scrub, dry farmland and habitation over a good deal of its range, often becoming very tame. The species is found in much of sub-Saharan Africa, Saudi Arabia, Iran, Afghanistan, Pakistan and India. It is also found in Israel, Lebanon, Syria, the UAE and Turkey (these populations may be derived from human introductions). Laughing doves are mostly sedentary but some populations may migrate. Birds ringed in Gujarat have been recovered 200 km north in Pakistan and exhausted birds have been recorded landing on ships in the Arabian Sea. Birds that land on ships may be introduced to new regions, acting as vectors of Newcastle disease virus (Wikipedia, 2014). It is also important to conduct further studies on the role of pigeons in the epidemiology of ND in Nigeria, since it has been reported that pigeons play a role in the epidemiology of ND in Europe (Alexander *et al*., 1984).

Nigeria's poultry population is estimated to be 137.6 million, with backyard poultry population constituting 84% (115.8 million) and 16% (21.7 million) of exotic poultry, with a higher percentage of this poultry raised for subsistence production (FDLPCS, 2006).Poultry production in Nigeria is peculiar having developed from the basic rural free range rearing of local chickens to the rearing of exotic or genetically improved chickens in the space of the last three decades(NVRI, 2004). The urbanization process in Nigeria has not made adequate provisions for farms and livestock as the cities and towns overgrow with haphazard residential buildings (SAHEL, 2015). Consequently in Zaria poultry production has been both intensive and extensive. Intensive poultry production in Zaria and its environs has developed significantly with large scale farms housing hundreds of thousands of birds. These farms are mainly run by retired to civil servants or military officers that have occupied one top position or the other in past regimes of government. Government farms like the A.B.U. farms Shika, and NAPRI-Shika also contribute to the poultry production in Zaria (personal communication).

Extensive poultry production in Nigeria especially Zaria, developed as backyard poultry in the poorly planned urban areas and as free-range in the rural areas. Nonetheless backyard poultry and free-range poultry meet the chicken meat and egg demand for citizens of Zaria. Staff of the Ahmadu Bello University have ventured into backyard poultry farming over the last two decades, providing employments and income for housewives of staff, absorbing villagers from neighbouring host communities (like Bomo, Samaru and Giwa) as hired poultry hands, and meeting the demand for eggs and chicken for Staff of the University and the host community, Samaru (personal communication with backyard poultry farmers in the study area). This is made possible because staff of A.B.U. Zaria resident in Areas A, BZ, F, E, G, C, Silver Jubilee, and H have one or more of their neighbours rearing poultry by the backyard method (Nawathe *et al.*, 1987).

Nevertheless, backyard poultry farming in Zaria is not without challenges top of which include getting high quality feed at affordable prices, and disease, especially ND which lowers production and causes economic loss. Though there is ready access to professional help from veterinarians and animal scientists from the university, NDis still plagues backyard poultry (personal communication with backyard poultry farmers). Most, if not all, backyard poultry farmers in the residential areas of A.B.U. Zaria, follow a vaccination schedule against ND, yet outbreaks of ND have been known to wipe out entire flocks or result in a drop of egg production (personal communication). This may suggest thatENDV may be introduced into the microcosm of backyard poultry farms. This could be through many sources including contaminated feed from the source or during storage, flock replacement, local chickens/free-range, vaccination failures, incursion of wild birds in poultry houses especially the laughing doves and feral pigeons, manure haulers, egg and broiler middle-men buyers, and hatchery (Margaret, 2006).

CHAPTER THREE

MATERIALS AND METHODS

3.1 Study Area

The study areas fall within latitude 11°10’38”N, longitude 7°37’43”E and latitude 11°8’18”N, longitude 7°41’3”E, occupying a land area of 26.59 Km^2. The residential areas used for this study were selected by convenience and comprised of the residential areas in Zaria especially the staff quarters of A.B.U. Samaru- Zaria. These residential areas were grouped as follows: Area BZ, Area F, Area E, Area G and Area C as cluster-1 (C1); Area H, silver jubilee and Palladan as cluster-2 (C2), and the Samaru live bird market (SLBM) in Zaria, Kaduna State (Figure 3.1). Birds from the Samaru live bird markets come from the Samaru and environs. Trapped doves from as far as Bakori in Katsina state and Gwari in Abuja were also brought and sold in the Samaru live bird market according to Mallam Yahaya who sells poultry and wild birds at the live bird market. Areas BZ, F, E, C, G, Silver jubilee and H are full of tall trees and lots of laughing doves and pigeons have made the wooded area their home range. These residential areas have some residents who rear broilers or layers by the backyard poultry method housing on the average 300 birds. There are two major live bird markets and chicken slaughter slabs in Zaria, one at the Samaru main market and the other at the Sabon gari general market. Trapped doves, feral and domesticated pigeons are housed together with chickens, ducks, guinea fowls, turkeys and quails at the live bird markets for sale to the public.

There is an Avian Clinic of the Veterinary Teaching Hospital and a Faculty of Veterinary Medicine, Ahmadu Bello University, Samaru- Zaria. Poultry farmers in the twoclusters(C1 and C2) and live bird traders in the Samaru live bird market (SLBM) can readily access professional veterinary services from these veterinary facilities. Though there is an Avian Influenza surveillance and rapid response team of the Avian Clinic established following the 2007 outbreak of avian influenza (HPAI), there is no exotic Newcastle disease (END) response unit even though the most important disease of poultry in Zaria is Newcastle disease (ND).

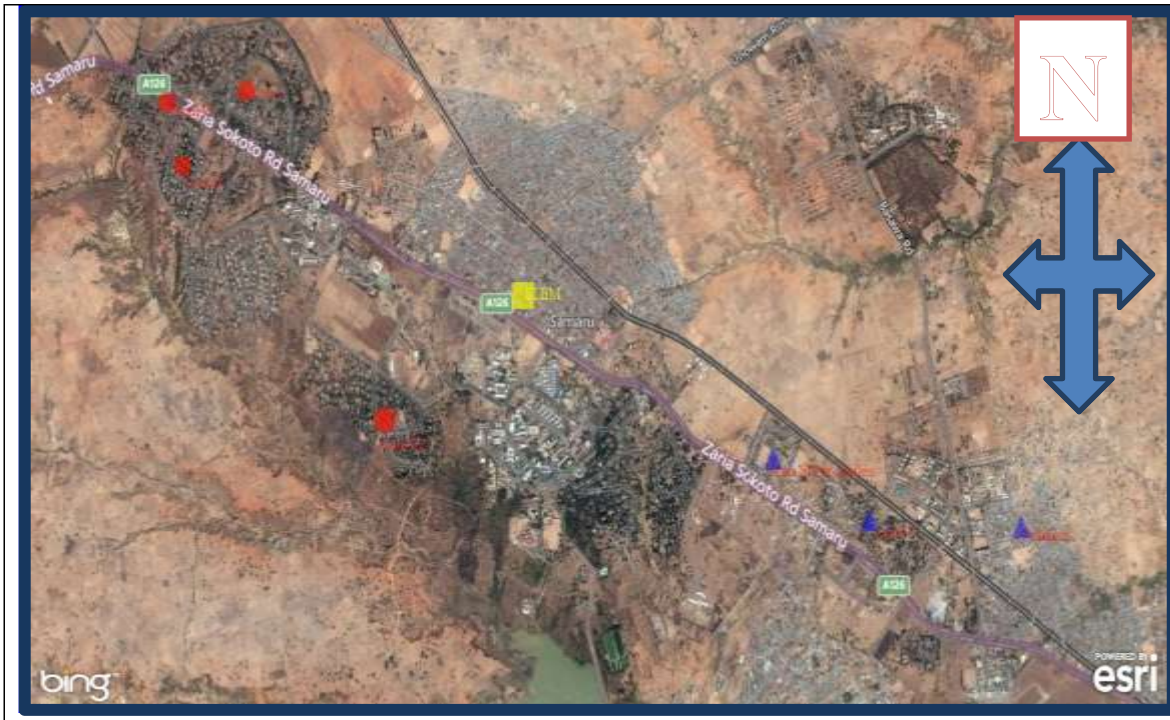
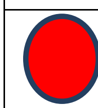
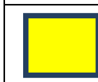
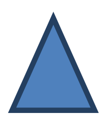


Figure 3.1 Satellite map of Samaru Zaria.

Source: Environmental Systems Research Institute

Horizontal Scale: 1cm: 409 m

Vertical Scale: 1cm: 480 m

Symbol	**Interpretation**
	Cluster 1: Areas BZ, E, G and C
	Samaru Live Bird Market (SLBM)
	Cluster 2: Area H, Silver Jubilee and Palladan
N	North

3.2 Study Design

A cross sectional study was carried out involving the survey of doves trapped from the premises of backyard poultry farmers in Areas BZ, E, G and C (Cluster 1), and areas H, Silver Jubilee of A.B.U. Staff quarters, and Palladan (Cluster 2), and from the Samaru live bird market (SLBM). Also,pretested semi-structured questionnaires were issued to backyard poultry farmers to assess the risk factors that may favour the outbreak of END from doves in their poultry farms.

3.3 Sampling and Sample Collection

3.3.1 Sample size

A total of sixty-seven laughing doves were trapped and sampled from clusters 1 and 2 and the Samaru live bird market. While twenty-five chickens from backyard poultry farms were sampled from the residential areas in clusters 1 and 2. This was subject to the number of doves that were trapped during the six month survey period from September 2014 to February 2015. Thus a sum total of 184 oropharyngeal and cloacal swabs were collected and tested. We purposed to sample at least ten doves and five chickens from each residential area.

3.3.2 Trapping of laughing doves.

Three locally constructed bamboo cages with apparently healthy laughing-doves and grains of guinea corn and millet, placed around backyard poultry farms, were used to lure and trap wild laughing doves in the study area (Plate I). Best trapping successes were obtained between 10am – 12 noon and 4pm – 6pm; times at which laughing doves foraged from the ground (Plate VIII). Between 12 noon – 4pm doves were either watering themselves or resting high up in the trees (Plate IX).

Each dove trapped and sampled was tagged by clasping non-corroding metallic colour-coded bracelets and rubber bungs on their shanks. Each residential area had a different colour code: Area BZ-green, Area E/G-blue, Area C-golden, Silver Jubilee and Area H-red/pink, and Palladan-silver; while subject doves from the Samaru live bird market were marked on their shanks with a black indelible ink. This was to prevent double sampling of subjects and in a follow up future study assess whether laughing doves migrate from one residential area to another area or were limited to the areas in which they were trapped initially (Plate III).

3.3.3 Sample collection

Sixty-seven pairs of oropharyngeal and cloacal swabs samples were collected from laughing doves. Each dove trapped, ringed and sampled was released back into the wild but was not sampled again even if re-trapped. Fourteen, twenty-eight and twenty-five doves were sampled from clusters 1, 2 and the Samaru live bird market respectively. A similar collection was done on five chickens conveniently selected from three out of four residential areas in cluster 1 and two out of three residential areas in cluster 2, making a total of twenty-five pairs of samples (Plate II). Oropharyngeal and cloacal swabs from doves trapped close to backyard poultry farms and the Samaru live bird market were eluted in 2.0 ml of viral transport medium in screw cap tubes, and frozen at -20^{o}C till analysis. Dead doves picked up from premises of backyard poultry farmers were necropsied (Plate VII).

3.3.4 Preparation of washed chicken red blood cells

Whole blood was collected into EDTA-coated tubes from apparently healthy local chickens with no known history of ND vaccination or clinical disease. The blood was washed thrice by centrifugation with dextrose gelatin veronal (DGV) solution (pH 7.4), and 10%, 1% and 0.1% suspensions of washed chicken red blood cells were prepared and stored at 4^{o}C till needed (Sally, 2002), (See Appendix V).

3.3.5 Antigen

Newcastle disease virus antigen was obtained by reconstituting commercially sold Newcastle disease LaSota vaccine (100 doses)in 20 ml of distilled water and using it fresh for the HI test (OIE, 2000;Sa'idu *et al.,* 2004; Wambura, 2010). The antigen had a titre of 1:256 with a 4HAU of 64.

Sterile PBS was prepared with pH of 7.4 to serve as diluent.

3.3.6 Positive control serum

Hyper-immune serum with antibodies to ND was obtained by raising ten broilers to 8 weeks of age, and vaccinating them at week 1, 3, 6 and 8 with LaSota vaccine. Ten millilitres (10mL) of whole blood was collected from these ten broilers at 10 weeks of age, pooled together, kept to stand and allowed to coagulate to obtain the serum (Plate IV). Serum was decanted into a screw cap sterile container and stored frozen at -20^{o}C (Sally, 2002). The HI titre was determined to be 1: 64.

Sterile PBS with pH 7.4 served as diluent for negative control which was blank of negative serum.

3.4 Laboratory Analysesfor Detection of Newcastle Disease Virus

3.4.1 Haemagglutination Assay and Haemagglutination Inhibition assay

Each oropharyngeal and cloacal swab was subjected to HA and HI tests in U-shaped micro-well titre plates. To each drop (0.02 ml) of test sample placed in a well, one drop of one percent (1%) washed chicken red blood cells was added. The sides of the plate were tapped lightly, and then the plate covered with a piece of paper, and left to stand for 30-45 minutes. The result was read as positive for haemagglutination if a diffuse mat of red blood cells was observed in the well and negative if a button of clumped red blood cells settled at the base of the well. These were compared with the reactions of the negative (RBC and PBS only) and positive (RBC, La Sota NDV, and PBS) control wells. Then 0.02 ml of positive control serum was added to each haemagglutination positive well and the positive control wells to observe for inhibition of haemagglutination. HI positive samples transformed from a diffuse mat of red blood cells to a button of clumped cells at the bottom of such a well while HI negative samples remained as diffuse mats of red blood cells (Sally, 2002) (Plate VI). This was compared with the positive and negative control wells.

3.4.2 RBC adsorption-de-adsorption concentration of Newcastle disease virus

Prior to RNA extraction and RT-PCR, NDV was concentrated by adsorption and de-adsorption of the viral particles to and from washed red blood cells respectively (Jianzhong and Chengqian, 2011).This was done by centrifuging 1ml of oropharyngeal or cloacal swab HI positive samples in a micro-centrifuge tube for 10 minutes at 12,000 rpm twice.Next 300 µl of supernatant was transferred into a new centrifuge tube and incubated with 50 µl of washed chicken RBC, then spun at 150 rpm for 30 minutes at room temperature to adsorb NDV to RBC (adsorption step).The mix was further spun at 2,110 rpm for 5 minutes at room temperature to concentrate the suspended RBC into a pellet at the base of the tube. Supernatant was decanted. The pellet of RBC with adsorbed NDV was re-suspended in 300 µl of PBS and then 50 µl of 5mM EDTA and 50 µl of beta mercaptoethanol were added to the mix and incubated at 37ºC for 5 minutes (de-adsorption step).The tube was spun at 2,400 rpm for 5 minutes to separate NDV from RBC.Finally, 200 µl of the NDV-rich supernatant was pipetted for RNA extraction.

3.4.3 RNA extraction

The concentrated 200 µl of NDV-rich test samples were subjected to RNA extraction using BIONEER AccuPrep® Viral RNA extraction kit. To every 200 µl NDV-rich test sample, 400µl of binding buffer (VB-which chaotropic salt isopropanol) were added, and then transferred into 1.5ml micro-centrifuge tubes and vortexed for 5 seconds. The tubes were incubated for 10 minutes at room temperature. Then 100 µl of isopropanol was added to the tube, lightly vortexed for 5 seconds, and then spun for 10 seconds. The binding column (in the presence of chaotropic salt-isopropanol, RNA is bound to glass fibers fixed in a column) was fitted into the 2 ml collection tube. The liquid was transferred into the binding column. The lids were closed carefully and centrifuged for 1minute at 8,000 rpm. Following centrifugation, the binding column was transferred to another 2 ml collection tube. Then 500 µl of washing buffer 2 (W2: 10 mM imidazole, 0.3 M sodium chloride, 50 mM sodium phosphate, pH 8.0) was added and centrifuged for 1 minute at 8,000rpm. After centrifugation, the binding column was transferred to 2 ml collection tube. Thereafter, 500 µl of washing buffer 2 was added and centrifuged for 1 minute at 8,000 rpm. The collection tube was spun down once more at 13,000 rpm for 1 minute to remove ethanol completely. The binding column was transferred to a 1.5ml collection tube, 50µl of elution buffer (EL: 250 mM imidazole, 0.3 M sodium chloride, 50 mM sodium phosphate, pH 8.0) was added, and allowed to stand for 1 minute to allow the buffer permeate the column. Eluted RNA was retrieved by spinning down at 8,000rpm for 1 minute and used directly for RT-PCR.

3.4.4 Reverse transcription – polymerase chain reaction (RT-PCR) test

RNA extracted from fifty-three of theHI positive samples were subjected to reverse transcriptase polymerase chain reaction(RT-PCR) technique using the primer set NDV-F 4829 (5'-GGTGAGTCTATCCGGARGATACAAG-3') and NDV-R 5031 (5'-TCATTGGTTGCRGCAATGCTCT-3'). These primers amplified the Fusion-gene segments of NDV antigen that coded for lentogenic, mesogenic or velogenic NDV.

The PCR reaction mix per extracted RNA sample contained 13 µl RNase-free water, 50 µl PCR Buffer 2 ($MgSO_4$ 2.4 mM; dNTPs 1.6 mM), 1 µl Primer NDV-F 4829, 1µlPrimer NDV-R 5031, 1 µl Enzyme mix (Taq polymerase enzyme) making a total reagent volume of 45µl. The mixture was vortexed for a few seconds then transferred into 0.2ml PCR tubes. Next, 5 µl of extracted RNA was added making a final volume of 50 µl. Reverse transcription and amplification of cDNA was achieved following optimization of cycling conditions using NDV RNA extracted from LaSota vaccine which also served as positive control. Placing the reaction mix in the thermocycler, cDNA was produced, by heating to

42°C for 60 minutes followed by raising the temperature to 94°C for 5 minutes to inactivate the enzymes. cDNA was amplified in 40 cycles consisting of 95°C for 15 seconds, 49°C for 30 seconds, and then 72°C for 30 seconds. After the 40 cycles enzymes were inactivated by maintaining the temperature at 72°C for 7 minutes. Amplicons were stored at 4°C till use.

3.4.5 Agarose gel electrophoresis

Amplicons were transferred into the wells of a 2% agarose gel for gel electrophoresis. The results were visualized after gel electrophoresis as follows: Lentogenic NDVappeared as 2 bands of approximately 135 bp and 67 bp while mesogenicor velogenic NDV appeared as a single band of approximately 202 bp.

3.5 Assessment of Biosecurity

Fifty five backyard poultry farmers in the residential areas under study who consented were interviewed using a pretested structured questionnaire by face-to-face interview. The questionnaire was pretested by administering to ten randomly selected backyard poultry farmers in Area BZ (R1) of the study area, and interviewing the first five with the questionnaire and then modifying ambiguous questions before testing it again on the second set of five. Every third house was sampled. The responses from these ten questionnaires were entered into SPSS data package and test for reliability statistics obtained a Cronbach's alpha of 0.804 which showed high homogeneity. The poultry facility of each respondent was inspected to verify responses on farm management practice (Plate VIII) and biosecurity measures (Plate IX).

The questionnaire had four sections: a preliminary section and three main sections A, B and C. For section A on assessment of biosecurity measures, a score of 7 to 10 out of 10 meant good biosecurity measures in place, while a score of 4 to 6 out of 10 meant fair biosecurity, and finally any score from 0 to 3 out of 10 meant poor biosecurity. For section B on assessment of ND outbreak, a score of 9 to 11 out of 11 impliedoutbreak of END, while a score of 5 to 8 out of 11 meant endemicity of END, and any score from 0-4 out of 11 meant sporadicity of END. While for section C on assessment of management practices a score of 7-8 out of 8 meant good management practice, while a score of 4 to 6 out of 8 meant fair management practice, and any score from 0 to 3 out of 8 meant poor management practice (Appendix I).

3.6 Data Analyses

Percent positives and frequency tables were calculated and drawn for the prevalence rates obtained by HA and HI tests and detection rates by RT-PCR tests on oropharyngeal and cloacal swabs obtained from the chickens and data from the questionnaire survey. Association between the categorical independent variables and outbreak of ND were assessed by chi-square analysis or by Fisher's exact test. Crude odds ratios (OR) and 95% confidence intervals (CI) on the odds ratios were calculated using bivariate logistic regression. P-values less than 0.05 indicated statistically significant associations.

Odds ratio values less than one showed the factor was protective while OR values equal to one showed the factor was not associated with the outbreak of ND and OR values greater than one meant the factor was associated with outbreak of the ND.

3.7 Public Health Action

Feedback in the form of information pamphlets on the need to improve biosecurity measures and how to prevent outbreak of ND and cross infection between doves and chickens were distributed to the backyard poultry farmers in Samaru Zaria. Farmers were encouraged to set up poultry farmers associations in the different residential areas so as to harmonize immunization against ND and improve biosecurity practices. This feedback was based on the major findings from the study (Appendix IX).

Plate I: Trapping of laughing doves with baited locally crafted bamboo traps.

Plate II: Collection of oropharyngeal swab from a trappedlaughing dove.

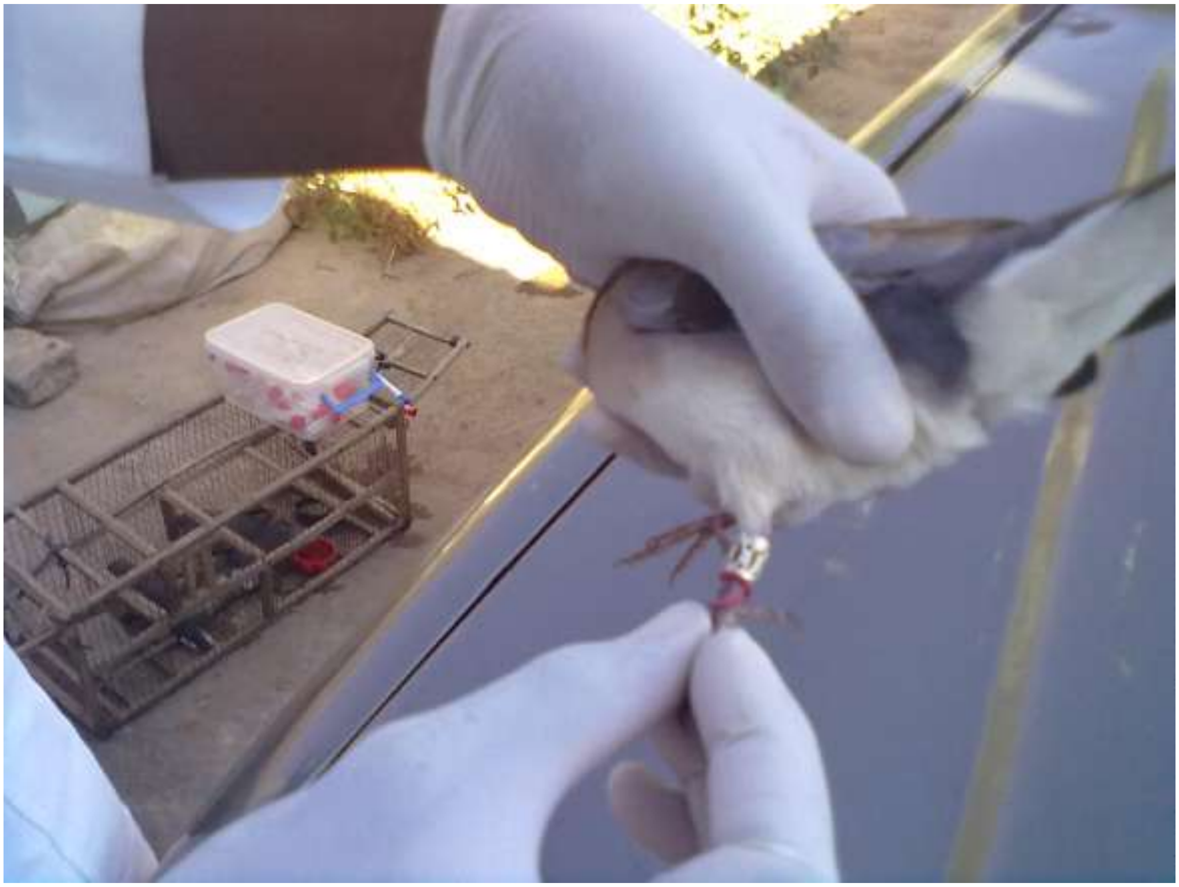


Plate III: Colour code tagging of a trapped and sampled dove with a metallic bracelet and rubber band.

Plate IV: Harvesting NDV hyper immune serum from a primed broiler.

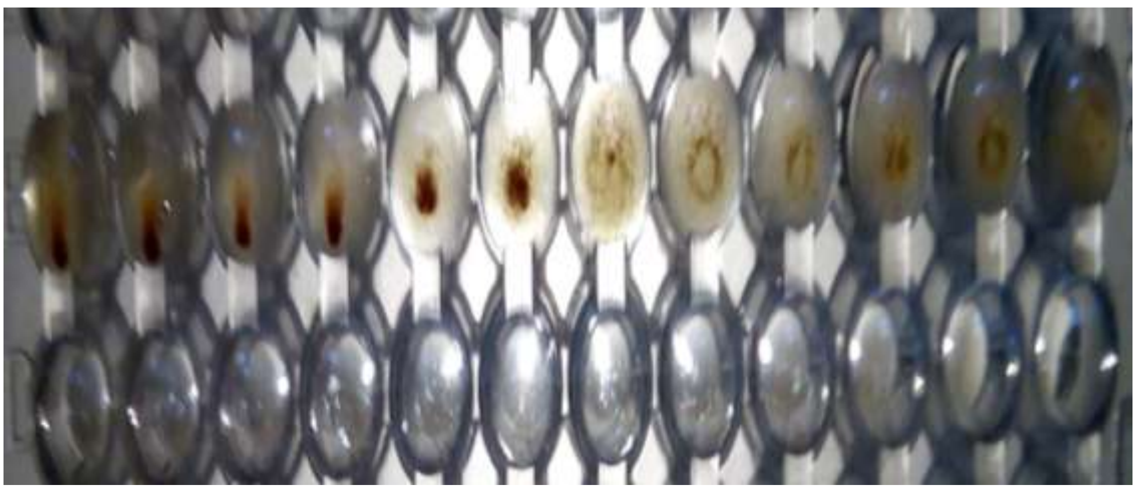


Plate V: Determination of HI titre (1:64) of the hyper immune serum.

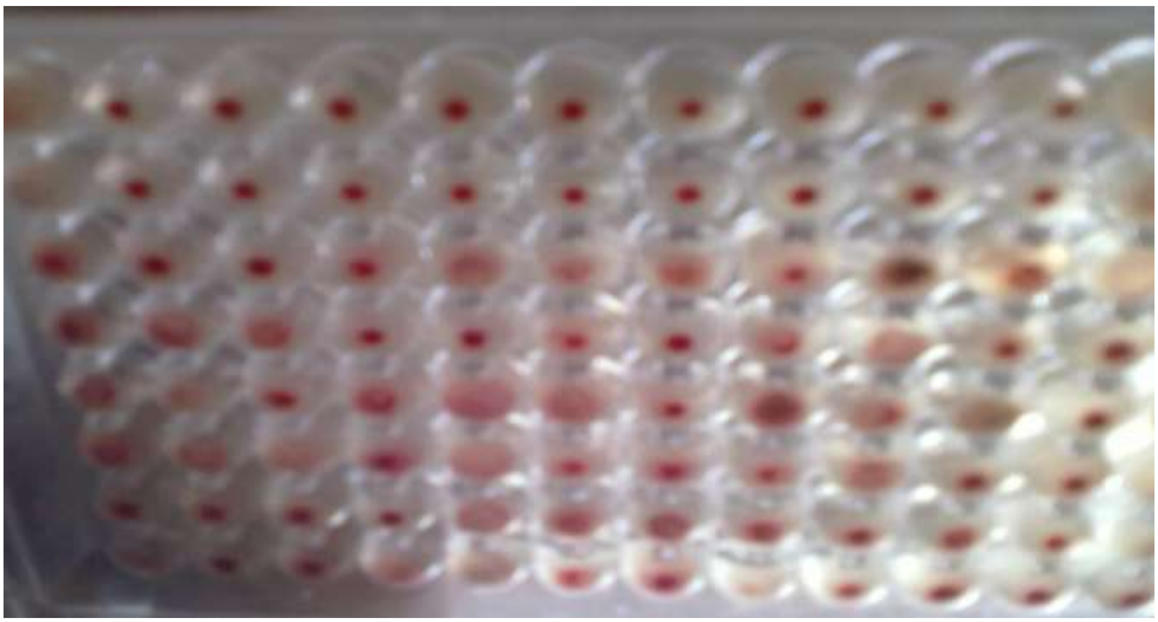


Plate VI: Screening of samples by haemagglutination test.

Plate VII: Postmortem of a dead dove found in the premises of a backyard poultry farmer. (Congested carcass and viscera).

Plate VIII: Poor disposal of spent poultry litter (top) and close contact between chickens and pigeons (bottom).

Plate IX: Laughing dove perched on an electric pole (left), electric cable (right) and landing on a rooftop (bottom).

CHAPTER FOUR

RESULTS

4.1 Detection of Newcastle Disease Virus

Out of 134 oropharyngeal and cloacal samples from laughing doves examined, 88 (65.7%) of them showed haemagglutination, 37 (42.1%) were positive for NDV by haemagglutination inhibition assay. Twenty one (56.8%) of the 37 HI positives were positive by conventional RT-PCR analysis (Plate X). Detection rate was higher from cloacal swabs (45.7%) than from oropharyngeal swabs (38.1%) of laughing doves by HI assay. However by RT-PCR analysis of HI positives, detection was higher from oropharyngeal swabs (68.8%) than from cloacal swabs (47.6%) (Table 4.1). Unlike the dove samples, there was higher detection rate in oropharyngeal (75%) than cloacal (63.6%) samples from chickens by HI assay while by RT-PCR there was a higher detection from cloacal samples (28.6%) than from oropharyngeal swabs (22.2%) (Table 4.2). Detection of NDV from doves by RT-PCR was highest in Samaru live bird market (SLBM) than from those in the residential areas (C1 and C2) (Table 4.3). While detection of NDV by RT-PCR from chickens sampled was highest in cluster 1 (Table 4.4). Twenty-five (25) samples were positive by RT-PCR consisting of 14 (one chicken and 13 dove samples) mesogenic or velogenic and 11 lentogenic pathotypes. Of the 13 mesogenic or velogenic dove samples, 7 were from the Samaru live bird market (SLBM), 2 from cluster 2 (C2) and 3 from cluster 1 (C1). Eight of the 11 lentogenic NDV were from doves and 3 from chickens. Six out of the 8 lentogenic NDV from dove samples were from the Samaru live bird market (SLBM), while 2 out of the 3 lentogenic NDV from chicken samples were from cluster 1 (Table 4.5).

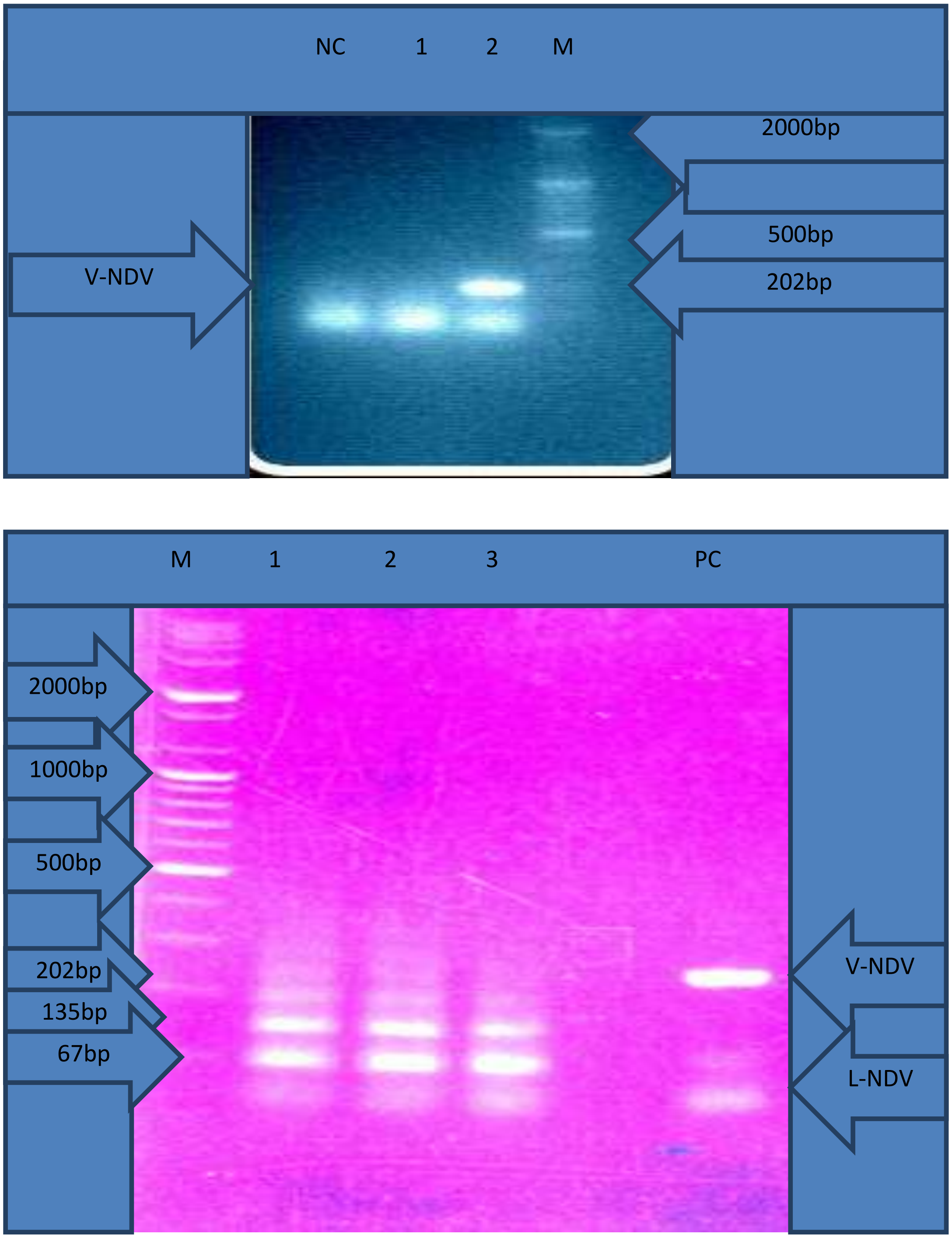


Plate X: Gel Electrophoregram of RT-PCR products from dove swabs from clusters 1 and 2 and the Samaru live bird market, Zaria, Nigeria.

Keys: M=Marker; 1, 2 and 3=dove samples from cluster 1, 2 and the Samaru live bird market; PC= Positive control;NC=Negative control; bp=base pair; V-NDV=Velogenic Newcastle disease virus; L-NDV=Lentogenic Newcastle disease virus. (Swabs 1 and 3 were oropharyngeal swabs while swab 2 was a cloacal swab).

Table 4.1: Detection of Newcastle disease antigen in laughing doves trapped around backyard poultry farms and the live bird market sampled in Samaru, Zaria, Nigeria.

Subject	**Swab sample type**	**No. tested**	**HA positive (%)**	**HI positive (%)**	**RT-PCR positive (%)**
Dove	Oropharyngeal	67	42(62.7)	16(38.1)	11(68.8)
Dove	Cloacal	67	46(68.7)	21(45.7)	10(47.6)
Totals		**134**	**88(65.7)**	**37(42.1)**	**21(56.8)**

Keys:

HA-Haemagglutination assay, HI-Haemagglutination Inhibition, RT-PCR –Reverse transcriptase polymerase chain reaction.

Table 4.2**:** Detection of Newcastle disease antigen in chickens frombackyard poultry farms sampled in Samaru, Zaria, Nigeria.

Subject	**Swab type**	**No. tested**	**HA positive (%)**	**HI positive (%)**	**RT-PCR positive (%)**
Chicken	Oropharyngeal	25	12(48.0)	9(75.0)	2(22.2)
Chicken	Cloacal	25	11(44.0)	7(63.6)	2(28.6)
Totals		**50**	**23(46.0)**	**16(69.6)**	**4(25.0)**

Keys:

HA-Haemagglutination assay, HI-Haemagglutination Inhibition, RT-PCR –Reverse transcriptase polymerase chain reaction.

Table 4.3: Detection of NDV in laughing doves using haemagglutination, haemagglutination inhibition and reverse transcriptase – polymerase chain reaction testsaccording to sample area sub-units, in Samaru-Zaria, Nigeria.

Subject	Area	Sample type	No. tested	HA positive (%)	HI positive (%)	RT-PCR positive (%)
Dove	Region 1	OPS/CS	28	23(82.1)	9(39.1)	4(44.4)
Dove	Region 2	OPS/CS	56	41(73.2)	12(29.3)	4(33.3)
Dove	SLBM	OPS/CS	50	24(48.0)	16(66.7)	13(81.3)
Totals			**134**	**88(65.7)**	**37(42.0)**	**21(56.7)**

Keys:

OPS/CS – Oropharyngeal swab and cloacal swab; Cluster 1-Areas BZ, C, E and G; Cluster 2 – Areas H, Silver Jubilee and Palladan; SLBM – Samaru live bird market, HA-Haemagglutination assay, HI-Haemagglutination Inhibition, RT-PCR –Reverse transcriptase polymerase chain reaction.

Table 4.4: Detection of NDV in chickens from backyard poultry farms using HA, HI and RT-PCR tests according to sample area sub-units, in Samaru-Zaria, Nigeria.

Subject	**Area**	**Sample type**	**Number tested**	**HA positive (%)**	**HI positive (%)**	**RT-PCR positive (%)**
Chicken	Cluster 1	OPS/CS	30	15(50.0)	9(60.0)	3(33.3)
Chicken	Cluster2	OPS/CS	20	8(40.0)	7(87.5)	1(14.3)
Totals			**50**	**23(46.0)**	**16(69.6)**	**4(25.0)**

Keys:

OPS/CS – Oropharyngeal swab and cloacal swab; Cluster 1-Areas BZ, C, E and G; Cluster 2 – Areas H, Silver Jubilee and Palladan; HA-Haemagglutination assay, HI-Haemagglutination Inhibition, RT-PCR –Reverse transcriptase polymerase chain reaction.

Table 4.5: Pathotype of Newcastle disease virus detected in subjects based on gel electrophoretypingin Samaru - Zaria, Nigeria.

Subject	**Area**	**Sample**	**RT-PCR positive (%)**	**Lentogenic NDV (%)**	**Velogenic NDV (%)**	**Mixed Pathotype (%)**
Dove	Cluster 1	OPS/CS	4(44.4)	1(25.0)	3(75.0)	0
Dove	Cluster2	OPS/CS	4(33.3)	1(25.0)	2(50.0)	1(25.0)
Dove	SLBM	OPS/CS	13(81.3)	6(46.2)	7(53.8)	0
Chicken	Cluster1	OPS/CS	3(33.3)	2(66.7)	1(33.3)	0
Chicken	Cluster 2	OPS/CS	1(14.3)	1(100)	0	0
Total			**25(47.2)**	**11(44)**	**13(52)**	**1(4)**

Keys:
OPS/CS – Oropharyngeal swab and cloacal swab; Cluster 1-Areas BZ, C, E and G; Cluster 2 – Areas H, Silver Jubilee and Palladan; SLBM – Samaru live bird market, RT-PCR – Reverse transcriptase polymerase chain reaction, NDV-Newcastle disease virus.

4.2 Assessment of Biosecurity Measures

Though respondents had set up some biosecurity measures (see Appendix XI) for their backyard poultry farms 85.5% of them had no functioning germicide footbath; 98.2% had trees/electric poles and cables close to their premises; 52.7% reportedly spilled poultry feed on the ground; and 27.3% sundried grains/flour on the ground in their premises (Table 4.6). There were associations between owner's reported outbreak of ND and lack of functioning germicide footbath (OR 3.28, 95% CI 0.37-29.12), spilled poultry feed (OR 1.17, 95% CI 0.36-3.74) and sun drying of grains/flour (OR 1.93, 95% CI 0.46-8.03) though none was statistically significant (Table 4.7).

4.3 Assessment of Newcastle Disease Outbreak

Of the 55 respondents interviewed 60% reported outbreak of velogenic ND even though 85% of them reported low mortality during such outbreaks. About 90.9% of respondents correctly intervened (sought veterinary assistance, separated apparently healthy birds from sick ones, replaced litter, and vaccinated the flock with La Sota Newcastle disease vaccine) during outbreak of ND in their farms. About 29.1% incurred maximal financial loss as a result of velogenic Newcastle disease with respondents unable to recover running costs. About 75.5% of respondents had reported non-vaccination of their flocks of chickens in the last four weeks prior to reported outbreaks of ND. About 67.3% of respondents reported sudden mortality of chickens in outbreaks of ND (Table 4.8). Respondents' reported type of ND outbreak, financial loss incurred, recent vaccination against ND, neighbouring farmers affected by the outbreak, major clinical signs observed in sick birds, postmortem lesions observed in dead birds and occurrence of sudden deaths were associated with outbreak of Newcastle disease (Table 4.9).

4.4 Assessment of Farm Management Practices

About 72.7% of respondents poorly disposed spilled/soiledpoultry feed (doves could access the rubbish heaps behind the backyard poultry house) and 45% poorly disposed carcasses of dead birds from their flocks. Interestingly 96% of respondents reported that laughing doves had no access to poultry feed (Table 4.10). Inaccessibility of laughing doves to poultry feed in backyard poultry farms was significantly protective against ND (OR 0.69, CI 0.59-0.83) (Table 4.11).

4.5 Monthly Distribution of Reported Newcastle Disease Outbreak by Respondents

Newcastle disease was mostly reported in the cold dry season and the peak of the rainy season in Zaria. November had the highest peak for the cold dry season (November to February) with a proportional prevalence rate of 30.76%. August with a proportional prevalence rate of 23.07% was the peak of reported outbreak of ND during the rainy season (May to October) (Table 4.12).

4.6 Distribution of Reported Newcastle Disease Based on Flock Size and Flock Type

The most common flock size was 300 birds as reported by 63.6% of respondents. Consequently the 300-bird flock size was reported to have the highest proportional morbidity rate of 53.8% but a flock specific rate of 60%. Of the 55 respondents, 34.5% raised broilers alone with a 33.3% proportional morbidity rate of Newcastle disease reported. About 27.3% of respondents raised layers alone with proportional morbidity rate of 25.6% and a flock specific rate of 66.7% while 27.3% of respondents raised layers and broilers together with a proportional morbidity rate of 35.9% and flock specific rate of 93.33% (Table 4.13).

4.7 Distribution of Backyard Poultry Farmers andPoultry Population in Samaru Zaria

Of the 55 respondents, 39 were from cluster 1 (Areas BZ, C, E, and G) while 16 were from cluster 2 (Areas H, Silver Jubilee and Palladan) (Table 4.14). The total number of birds reared by respondents in clusters 1 and 2 were found to be 20,447 birds and 2,358 birds respectively, amounting to 22,805 birds. Thus, the average flock-sizeswere 524.3 and 147.4 for cluster 1 and 2 respectively. The population of poultry in the Samaru live bird market was transient depending on demand and supply.

4.8 Assessment of Biosecurity and Farm Management in Relation to Outbreak of Newcastle Disease

Overall 55% of respondents had both good biosecurity measures in place and reported outbreak of ND was endemic in their farms respectively while 71% had fair management practice (Table 4.15).

4.9 Distribution of Doves Trapped and Sampled in Comparison to the Backyard Poultry Houses in the Study Area from September 2014 to February 2015

Cluster 1 (Areas BZ, G, E and C) had 50 backyard poultry houses whilecluster 2 (Silver Jubilee quarters, Area H and Angwan Fulani Palladan) had 25 poultry houses. The number of doves trapped and sampled in clusters1 and 2 were 14 and 28 respectively (Table 4.16).

Table 4.6: Distribution of respondents' use of biosecurity measures against outbreak of exotic Newcastle disease in Samaru-Zaria, Nigeria.

Factor	**Frequency (%) Yes**	**Frequency (%) No**	**Total (%) n**
A1. Germicide footbath	8(14.55)	47(85.45)	55(100)
A2. Intact chicken wire	45(81.82)	10(18.18)	55(100)
A3. Specific footwear per pen	43(78.18)	12(21.82)	55(100)
A4.Trees/pylons present	54(98.18)	1(1.82)	55(100)
A5. Visitors enter pen	12(21.82)	43(78.18)	55(100)
A6. Spilling of poultry feed	29(52.73)	26(47.27)	55(100)
A7. Grains/flour sundried	15(27.27)	40(72.73)	55(100)
A8. Good litter disposal	47(85.45)	8(14.55)	55(100)
A9. Feed stored indoors	53(96.36)	2(3.64)	55(100)
A10. Own pigeons/doves	7(12.73)	48(87.27)	55(100)

Table 4.7: Association between biosecurity factors and owner reported outbreak of Newcastle disease in backyardpoultry farms in Samaru-Zaria, Nigeria.

Factor	Present	Newcastle Disease		Chi sq.	P-value	Fisher's 2 sided	OddRatio	95% Confidence Ievel		Remark
		Yes	No					Lower	Upper	
A1.Germicide footbath	Yes	7	1			0.41	3.28	0.37	29.12	NS
	No	32	15							
A2.Intact chicken wire	Yes	31	14			0.71	0.55	0.10	2.95	NS
	No	8	2							
A3.Specific footwear	Yes	32	11	1.18	0.28	0.30	2.08	0.55	7.91	NS
	No	7	5							
A4.Trees/pylons	Yes	39	15			0.29				
	No	0	1							
A5.Visitors trespass	Yes	8	4	0.13	0.71	0.73	0.77	0.20	3.06	NS
	No	31	12							
A6.Spilled feed	Yes	21	8	0.07	0.79	1.00	1.17	0.36	3.74	NS
	No	18	8							
A7.Sundrying grains/flour	Yes	12	3	0.83	0.36	0.51	1.93	0.46	8.03	NS
	No	27	13							
A8.Good litter disposal	Yes	34	13	4.56	0.57	0.67	1.57	0.33	7.52	NS
	No	5	3							
A9.Feed stored indoors	Yes	38	15			0.50	2.53	0.15	43.17	NS
	No	1	1							
A10.Own pigeons	Yes	4	3			0.40	0.50	0.09	2.519	NS
	No	35	13							

Key:
NS-Not Significant

Table 4.8: Distribution of responses on assessment of indicators of outbreak of Newcastle disease in Samaru-Zaria, Nigeria.

Indicator	Description	Frequency (%)	Total (%)
B2.Type of ND	Lentogenic	22(40.00)	55(100)
	Velogenic	33(60.00)	
B3. Mortality	Low	47(85.45)	55(100)
	High	8(14.55)	
B4. Intervention	Correct	50(90.91)	55(100)
	Wrong	5(9.09)	
B5. Financial Loss	Minimal	39(70.91)	55(100)
	Maximal	16(29.09)	
B6. ND Vaccination	Not vaccinated	37(75.45)	55(100)
	Vaccinated	18(24.55)	
B7. Other farmers affected	Yes	13(23.64)	55(100)
	No	42(76.36)	
B9 Major signs	Mild ND	23(41.82)	55(100)
	END	32(58.18)	
B10. PM lesions	Mild ND	38(69.09)	55(100)
	END	17(30.91)	
B11. Sudden deaths	Yes	37(67.27)	55(100)
	No	18(32.73)	

Keys:

ND – Newcastle disease, END – Exotic Newcastle disease (velogenic Newcastle disease).

Table 4.9: Associations between factors that indicate exotic Newcastle disease and outbreak of Newcastle disease in backyard poultry farms in Samaru-Zaria, Nigeria.

Factor/Variable	Description	Newcastle Disease Yes	Newcastle Disease No	Fisher's 2 sided	Remark
B2.Type of ND	Lentogenic	6	16	0.00	Significant
	Velogenic	33	0		
B3. Mortality	Low	31	16	0.09	Not Significant
	High	8	0		
B4. Intervention	Correct	34	16	0.31	Not Significant
	Wrong	5	0		
B5. Financial Loss	Minimal	23	16	0.00	Significant
	Maximal	16	0		
B6. ND Vaccination	Yes	21	16	0.00	Significant
	No	18	0		
B7. Other farmers affected	Yes	13	0	0.01	Significant
	No	26	16		
B9 Major signs	Mild ND	7	16	0.00	Significant
	END	32	0		
B10. PM lesions	Mild ND	22	16	0.00	Significant
	END	17	0		
B11. Sudden deaths	Yes	37	0	0.00	Significant
	No	2	16		

Keys:
ND – Newcastle disease, END – Exotic Newcastle disease (velogenic Newcastle disease).

Table 4.10: Distribution of respondents' farm management practices that was associated with outbreak of exotic Newcastle disease in Samaru-Zaria, Nigeria.

Factor	FrequencyYes (%)	No(%)	Total (%)
C1. All-in-all-out policy	36(65.45)	19(34.55)	55(100)
C2. Clean pen premises	49(89.09)	6(10.91)	55(100)
C3. Hired hands don’t own birds	6(10.91)	49(89.09)	55(100)
C4. Effective waste feed disposal	15(27.27)	40(72.73)	55(100)
C5. Doves can’t access feed	53(96.36)	2(3.64)	55(100)
C6. Doves can’t access water	50(90.91)	5(9.09)	55(100)
C7. Good carcass disposal	30(54.55)	25(45.45)	55(100)
C8. Keep farm record book	27(49.09)	28(50.91)	55(100)

Table 4.11: Assessment of association between farm management practice factors and outbreak of Newcastle disease in backyard poultry farms in Samaru- Zaria, Nigeria.

Factor/Variable	Present	Newcastle Disease Yes	Newcastle Disease No	Odd Ratio	95% Confidence interval Lower	95% Confidence interval Upper	Remark
C1. All-in-all-out policy	Yes	26	10	1.20	0.38	4.03	Not significant
	No	13	6				
C2. Clean premises	Yes	36	13	2.77	0.50	15.49	Not significant
	No	3	3				
C3. Hired hands not to own birds	Yes	3	3	0.36	0.07	2.02	Not significant
	No/NA	36	13				
C4. Effective waste feed disposal	Yes	11	4	1.18	0.31	0.23	Not significant
	No/NA	28	12				
C5. Doves can't access feed	Yes	37	16	0.69	0.59	0.83	Significant
	No	2	0				
C6. Doves can't access water	Yes	36	14	1.71	0.26	11.38	Not significant
	No	3	2				
C7. Good carcass disposal	Yes	24	6	2.67	0.80	8.86	Not significant
	No	15	10				
C8. Keep farm record book	Yes	19	8	0.95	0.30	3.04	Not significant
	No	20	8				

Key:

NA-Not Applicable

Table 4.12: Distribution of outbreak of Newcastle disease by month as reported by poultry farmers in Samaru Zaria, Nigeria.

Variable	Month	Newcastle disease Yes	No	Total (%)	Proportional prevalence rate
Latest ND outbreak	NA	1	16	17(30.91)	2.56
	January	4	0	4(7.27)	10.25
	February	1	0	1(1.82)	2.56
	March	1	0	1(1.82)	2.56
	April	1	0	1(1.82)	2.56
	June	5	0	5(9.09)	12.82
	August	9	0	9(16.36)	23.07
	October	3	0	3(5.45)	7.69
	November	12	0	12(21.82)	30.76
	December	2	0	2(3.64)	5.12
	Total	39	16	55(100.00)	99.95

Key :

NA-Not Applicable

Table 4.13: Distribution of Newcastle disease (ND) outbreak by flock size and flock type as reported by backyard poultry farmers in Samaru-Zaria, Nigeria.

Variable	**Range/Type**	**Newcastle Disease**		**Proportional morbidity rate**	**Total Frequency (%)**	**Flock specific rate**
		Yes	**No**			
Flocksize	0-300	21	14	53.8	35(63.6)	60.0
	301-600	10	1	25.6	11(20.0)	90.9
	601-900	3	0	**7.7**	3(5.4)	100.0
	>900	5	1	12.8	6(10.9)	83.3
Total		**39**	**16**	**99.9**	**55(100.0)**	
Flock-type	Broilers	13	6	33.3	19(34.5)	68.4
	Layers	10	5	25.6	15(27.3)	66.7
	Layers/broilers	14	1	35.9	15(27.3)	93.3
	Local/crosses	2	2	5.1	4(7.3)	50.0
	Broiler/geese	0	1	0.0	1(1.8)	0.0
	Local/pigeons	0	1	0.0	1(1.8)	0.0
Total		**39**	**16**	**99.9**	**55(100.0)**	

Table 4.14: Distribution of poultry farmers, population of birds kept and Newcastle disease positivity by clusters in Samaru-Zaria, Nigeria.

Area	Number of Respondents	Number of birds kept	Average flock size per farmer	Number of samples tested.	HA Positivity (%)	HI Positivity (%)	RT-PCR Positivity (%)
Cluster 1	39	20447	524.3	30	15(50)	9(60)	3(33.3)
Cluster 2	16	2358	147.4	20	8(40)	7(87.5)	1(14.3)
Totals	55	22805	671.7	50	23(46)	16(69.6)	4(25)

Keys:

Cluster 1-Areas BZ, C, E and G; Cluster 2 – Areas H, Silver Jubilee and Palladan, HA-Haemagglutination assay, HI-Haemagglutination Inhibition, RT-PCR –Reverse transcriptase polymerase chain reaction..

Table 4.15: How respondents fared in biosecurity, outbreak of Newcastle diseaseand farm management practices in Samaru, Zaria, Nigeria.

Assessment	Cluster 1	Cluster 2	Total (%)
Biosecurity:			
Good	24	6	30(55)
Fair	17	8	24(44)
Poor	0	1	1(1)
ND outbreak:			
Epidemic	4	3	7(13)
Endemic	24	6	30(55)
Sporadic	12	6	18(32)
Management:			
Good	10	1	11(20)
Fair	26	13	39(71)
Poor	4	1	5(9)

Keys:

Cluster 1 comprised of Areas BZ, C, E and G.

Cluster 2 comprised of Area Silver Jubilee, Area H and Palladan.

Table 4.16: Distribution of doves trapped andbackyard poultry houses sampled in Samaru-Zaria, Nigeria from September 2014 toJanuary 2015.

Parameter/Study Areas	**C1**	**C2**	**SLBM**	**Total**
Number of Backyard Poultry houses	50	25	0	75
Number of Backyard Poultry houses Sampled	38	17	0	55
Number of Doves Trapped	14	28	25	67

Keys:
C1: Cluster 1(Areas BZ, G, C & E); C2: Cluster 2 (Areas Silver Jubilee, H, Palladan); SLBM (Samaru live bird market).

CHAPTER FIVE

DISCUSSION

This study was able to demonstrate that NDV was circulating in wild laughing doves and backyard chickens. Chantal *et al.* (2013) had reported that chickens were more often infected by NDV than other birds. They also reported that chickens from live bird markets were significantly more often positive for NDV than birds at commercial or backyard farms which contributed to the enzootic circulation of NDV. This is in agreement with findings from our study in which detection of NDV in doves was significantly higher in doves from the Samaru live-bird market than trapped wild doves from the residential areas in clusters 1 and 2. This could be attributed to the absence of biosecurity measures at the live-bird market. Doves trapped from within and outside Zaria were kept with chickens and other birds in small cages at the live bird market. This increased the possibility of infection between susceptible and infected birds shedding the virus due to the close proximity of birds in such small cages, poor hygiene, poor nutrition, transportation stress and overstocking. Residents from the two clusters 1 and 2 who buy live doves or chickens from the live–bird market and return home with them may help in the enzootic circulation of NDV in Samaru-Zaria. Chantal *et al.* (2013) explained that chickens in live-bird markets in Nigeria were sourced from both vaccinated (commercial or backyard poultry) and unvaccinated (free-range local chickens) flocks. Vaccination likely reduced the expression of clinical signs of Newcastle disease (Chantal *et al*., 2013). This did not necessarily suppress viral shedding from asymptomatic chickens (Chantal *et al*., 2013). The relatively high detection of NDV from apparently healthy backyard chickens from the two clusters 1 and 2 in this present study is not unexpected as farmers vaccinated their chickens as often as once every monthwith the La Sota NDV vaccine (personal communication with respondents). Chantal *et al.* (2013) had reported that outbreaks of ND in vaccinated flocks have been increasingly reported from Nigeria suggesting a suboptimal protection by vaccination. All NDV vaccine strains (genotypes I, II and III) are thought to protect against all virulent strains, except for some variant viruses that overcome vaccine protection (Chantal *et al.*, 2013). Challenge experiments with genotype XVIIa or XVIIIb strains after vaccination with the commonly used La Sota vaccine also confirmed that vaccination conferred efficient protection against clinical disease and mortality by these West African strains, although shedding was not inhibited for all animals (Chantal *et al.*, 2013). We may also consider poor vaccine quality, suboptimal vaccination or coinfections which may result in reduced vaccine efficacy (Chantal *et al.*, 2013).

In our study, detection of NDV from oropharyngeal swabs was higher than from cloacal swabs of chickens. This agreed with Haque *et al.* (2010) who reported that viral isolation rate from clinical samples was found highest in tracheal swabs (90%) compared to cloacal swabs (85%) and serum (65%). However, in this study the reverse was the case for samples from doves with detection rate higher in cloacal swabs than oropharyngeal swabs.
Our study also found out that NDV from doves were slightly more of the mesogenic or velogenic than the lentogenic pathotypes. This agrees with the findings from Seal and Brown (2001) who reported that PPMV-1 (NDV) from doves were typically mesogenic by ICPI in chickens. The duo also reported that PPMV-1 isolates from doves increased their virulence in chickens after passage and therefore represented a genuine threat to poultry production. Alexander (1997) recommended that isolation of a virulent strain of NDV from field samples required reporting to the Office International des Epizooties (OIE). With the detection of virulent or mesogenic NDV from laughing doves in Zaria in this study there is

need for reporting to the OIE by the Veterinary agencies of the Nigerian Government following standard reporting protocol.

Newcastle Disease virus could be detected by subjecting oropharyngeal swabs (OPS) and or cloacal swabs (CS) from laughing-doves and backyard poultry to haemagglutination assay (HA) as a screening test since NDV has haemagglutinating properties (Okwor *et al*., 2010). Subjecting positive haemagglutinating samples to haemagglutination inhibition (HI) assay using hyper-immune serum was able to narrow down the confirmatory detection of NDV. Running reverse-transcription polymerase chain reaction (RT-PCR) on such HI positives was able to confirm the presence of NDV RNA and classify NDV into lentogenic or mesogenic/velogenic pathotypes. The sensitivity of RT-PCR could be increased by concentrating the virus in swabs by a red blood cell adsorption-de-adsorption technique (Jianzhong and Chengqian, 2011). The higher detection of ND antigen from cloacal swabs than oropharyngeal swabs of laughing doves may be attributed to the fact that birds were primarily exposed to NDV by oronasal routes. Localization of the virus in the gastrointestinal tract is common in the lentogenic Newcastle disease (Jos *et al.*, 2011). Lentogenic NDV has a monobasic amino acid motif at the F-cleavage site 112 G-R/K-Q-G-R*L 117 which is cleaved extracellularly by trypsin-like proteases found in the respiratory and more so in the intestinal tract (Jos *et al.*, 2011). Alexander (1997) had reported that the NDV that was detected in pigeons was mostly lentogenic strains which could mutate to mesogenic/velogenic strains after passaging in chickens. Vaccination of chickens with LaSota vaccine produces a mild respiratory form of Newcastle disease (Sally, 2002) and may have influenced the detection pattern, with higher detection rates from oropharyngeal swabs than from cloacal swabs of chickens. This study reaffirmed that HA and HI tests can be used to detect NDV from field samples. Also other haemagglutinating avian pathogens not limited to Avian Influenza (H5N1) virus and Egg-drop syndrome adenovirus, and unlikely agents like *Mycoplasma gallisepticum, M. septicum* and Avian Pathogenic *Escherichia coli* (APEC) with haemagglutinating properties could be diagnosed similarly. In this study the HA positives that were not HI positive could be due to avian influenza virus (AIV) or any of the haemagglutinating pathogens mentioned previously.

Oladele *et al*. (1996) had reported that NDV had only been demonstrated in Nigerian pigeons by serological evidence. This study revealed that NDV could be demonstrated directly from oropharyngeal and cloacal swabs from doves and chickens. Subjection of such swabs to HA tests followed by HI tests demonstrated the presence of NDV. Conventional RT-PCR using the primer set NDV-F 4829 and NDV-R 5031 was able to pathotype the NDV demonstrated, from swabs that were both HA and HI positive, as either lentogenic and or velogenic. The ‘gold standard’ for diagnosis of NDV involves isolation and cultivation in embryonated chicken eggs, followed by HA, HI and ICPI which are labour intensive and time consuming (Alexander, 1998). The methods employed in this study used HA to screen field swab samples rapidly. HA positives were then subjected to HI test which was also easily done. And then samples that were both HA and HI positive were subjected to RT-PCR for pathotyping. To improve the sensitivity of RT-PCR the HA and HI positives were subjected to a red blood cell adsorption-de-adsorption concentration prior to RT-PCR which mimicked the results reported by Jianzhong and Chengqian (2011).

The use of HA, HI and RT-PCR seemed cheaper, faster, easier, convenient and feasible than the ‘gold standard’ for this study. There was also no need to sample the birds twice as we would have done if we depended on serology to differentiate between active infection and previous exposure.

The higher percentage of respondents who had no functioning germicide footbath compared to those who had is alarming. Respondents with germicide footbaths were 3.28 times more likely to recall an outbreak of Newcastle disease than those without. Those who had germicide footbaths did not make them functional. Respondents felt using specific footwear per pen was enough to check the contamination of pens from pathogens in their premises. Those who used specific footwear per pen were 2.08 times more likely to recall outbreaks of ND than those who did not. Respondents with intact chicken wire fencing that kept out doves from invading their poultry houses were 0.55 times less likely to recall outbreaks of ND than those with broken chicken wire. However respondents who spilled poultry feed and dried grains or flour in their premises were 1.17 and 1.93 times respectively more likely to recall outbreaks of ND than those who did not. Margaret (2006) and the USDA (2014) had recommended that optimum biosecurity plays a vital role in the prevention and spread of Newcastle disease. The poor implementation of and lax adherence to biosecurity measures by respondents in this study may have played a role in the detection of Newcastle disease virus in chickens.

Assessment of outbreaks of exotic Newcastle disease had shown that respondents’ recall of morbidity and mortality rates were not typical of velogenic Newcastle disease. Virtually all the respondents who recalled outbreak of Newcastle disease in their flocks claimed that there were occurrences of sudden death of chickens even though they had vaccinated their birds at least once every month. The atypical mortality rate could be ascribed to the high frequency of vaccination of flocks by respondents. This agrees with Chantal *et al*. (2013) where it was observed that vaccination against Newcastle disease in chickens reduced the expression of clinical signs but did not necessarily prevent shedding of the virus.

In terms of farm management practices, respondents who recalled that doves could not access their poultry feed were 0.69 times less likely to recall outbreaks of Newcastle disease than those where doves had access to poultry feed. Alexander (1997) had reported that there had been outbreaks of Newcastle disease in chickens in England in 1984 due to contamination of poultry feed with infected pigeon droppings. Dilaveris *et al*. (2007) also reported outbreak of Newcastle disease in chickens due to NDV of pigeon origin. Respondents need to improve on biosecurity in preventing wild birds, especially doves, from coming into contact with poultry directly or indirectly through fomites.

In a previous study Sa'idu *et al*. (2004) had reviewed cases presented to the avian clinic of the Ahmadu Bello University Veterinary Teaching Hospital (ABUVTH) from 1990-1999. They reported that the highest numbers of Newcastle disease recorded was during the dry season (January to March). They also observed that Newcastle disease was 3.4 times more likely to be reported in December than in November and January. Nwanta*et al*. (2008) had similarly observed that Newcastle disease occurred all year round but peaked from October to December. This they attributed to the aero-stability of NDV in the cold dry winds of the harmattan in Nigeria. In this study however, there were two peaks of Newcastle disease outbreaks as recalled by respondents. It was observed that November had the highest reported cases of Newcastle disease in the cold dry season. While in the wet rainy season August had the second peak of reported outbreak of Newcastle disease in Samaru-Zaria. This seasonal pattern observed could be attributed to the cold and windy nature of these two months which may have favoured the survivability and spread of NDV in the environment. The brightness of the sun is often lowered by the cloudy and hazy nature of the months of August and November respectively in Zaria. Lowered ambient temperature of 20^{o}C it has been reported favour the survivability of NDV in soil (Margaret, 2006). The August peak of reported Newcastle disease may also be due to the use of rainwater collected from rooftops that may have been contaminated with dove droppings to water chickens. Chantal *et al*. (2013) had also observed that seasonal demand for chickens was linked with increased incidence of Newcastle disease in Nigeria. In this study broilers were raised by respondents targeting the Christmas period in December. The broiler day-old chicks were normally stocked in November. Age-wise susceptibility may have been responsible for the broilers coming down with Newcastle disease within the first four weeks of life, hence the peak in November.

Flock size and flock type were also important in the distribution of reported outbreaks of Newcastle disease in this study. While Sa'idu *et al*. (2004) had observed that the highest outbreaks of Newcastle disease were recorded in flocks with 1-100 birds; we observed in this study that respondents who raised 1-300 birds recalled the lowest outbreaks of Newcastle disease. As the flock size increased the number of outbreaks increased.

Overstocking of poultry farms may have contributed to this pattern observed as respondents had limited backyard premises to adequately space out their pens.

Respondents who raised layers and broilers together reported the highest number of Newcastle disease outbreaks. Those who raised layers alone had the lowest outbreaks. The observed pattern may have been attributed to the age-wise susceptibility of chickens to Newcastle disease virus. Broilers were chicks with developing immunity so susceptible to Newcastle disease while layers were raised to adulthood with fully developed immunity and greater resistance to Newcastle disease virus infection. The layers may probably have been shedding the virus in the pen such that new broiler stock got exposed to NDV from layers before they could mount a strong immunity. Sa'idu *et al*. (2004) had observed that outbreak of Newcastle disease was 27.47 times more likely to occur in birds of 11-15 weeks of age than older birds in commercial poultry farms.

There were more respondents in cluster 1 than cluster 2 and this matched the number of chickens kept in the two regions respectively. The average number of chickens per respondent was therefore higher in cluster 1 than cluster 2. There was also a higher detection of NDV in chickens from cluster 1 than cluster 2. Newcastle disease virus spreads easily where there is a high population of chickens. Sa'idu *et al*. (2004) had however observed that farms with large flocks recorded less number of outbreaks. The difference between our observation and that of Sa'idu *et al*. (2004) could be that pens housing large flocks of backyard poultry farmers were not adequately spaced out while pens housing large flocks of established commercial farms were adequately spaced out.

The HA and HI tests on both oropharyngeal swab samples (OPS) and cloacal swab samples (CS) from chickens was one highly sensitive. However specificity was higher in OPS than in CS. It therefore implies that one is more likely to detect NDV from OPS using the HA and HI techniques than from CS. Newcastle disease is primarily a respiratory disease and so the virus may be more present in the oropharynx and larynx than in the cloaca as observed by Haque *et al*. (2010).

The scoring of respondents on biosecurity, outbreak of ND and farm management practice assessments indicated that cluster1 generally had better scores than cluster 2. Good and fair biosecurity and farm management practices resulted in sporadic and enzootic outbreaks of Newcastle disease respectively in the two clusters. This agreed with the findings from Chantal *et al*. (2013) who observed that birds from live-bird markets were significantly more often positive for NDV than birds from commercial or backyard poultry farms. This was attributed to the lower biosecurity and poor management of chickens in live-bird markets compared with those observed in backyard or commercial poultry farms.

Finally more doves were trapped and sampled in cluster 2 than cluster 1. The greater success of trapping doves in cluster 2 may be due to the fewer number of poultry houses present. This may have made the doves in cluster 2 congregate around the few poultry farms. It was thus easier to lure and trap a larger number of doves present in the area that scavenged for food in the premises of backyard poultry farmers. The reverse was observed in cluster 1 where though there were lots of doves, few doves congregated in the premises of the numerous houses with backyard poultry farms.

CHAPTER SIX

CONCLUSION AND RECOMMENDATIONS

6.1 Conclusion

From this study the following conclusions were made:

1. About 65.7% ofthe 134 oropharyngeal and cloacal swabs from laughing doves in Samaru-Zaria exhibited haemagglutination 42.1% of which was due to Newcastle disease virus.
2. About 38.1% of the 21 NDV detected in laughing doves in Samaru-Zaria were demonstrated to be lentogenic, 57.1% weremesogenic/velogenic while 4.8% were mixture of both lentogenic and velogenic NDV.
3. Poultry farmers with intact chicken wire barriers that kept out doves were 0.55 times less likely to recall an outbreak of NDthan farmers with broken chicken wire barriers.
4. Respondents who reported that doves could not access their poultry feed were 0.69 times less likely to recall an outbreak of NDin their farms when compared to those who reported that doves could access poultry feed.
5. The commonest index of outbreak of ND as recalled by respondents was sudden deaths of chickens overnight.
6. November and August were the months in which respondents recalled the highest outbreaks of NDin their flocks.
7. Backyard poultry farmers who raised over 300 birds and raised layers and broilers together reportedly had the highest outbreaks of ND respectively.

8. Good biosecurity and good management assessment scores resulted in sporadic outbreaks of ND in poultry farms as reported by respondents.

6.2 Recommendations

The following recommendations were made:

1. Backyard poultry farmers in the different residential areas should set up community poultry farmers associations to foster and harmonize prevention and control programmes against outbreak of Newcastle disease.
2. Poultry farmers in Samaru should give booster vaccinations against Newcastle disease in the months of June and September so as to confer immunity during the peak periods of outbreaks in the months of August and November.
3. Rainwater collected from rooftops should not be used untreated to water backyard poultry as it may be contaminated with faeces from infected doves shedding NDV.
4. Further studies should be directed towards screening laughing doves for haemagglutinating avian pathogens particularly Avian Influenza virus, Egg-drop syndrome adenovirus, and *Mycoplasma gallisepticum.*
5. For optimum detection of NDV by RT-PCR oropharyngeal and cloacal swabs should be analyzed as soon as they are obtained due to poor cold storage conditions and possible denaturing of viral RNA from incessant power outages in Zaria. Obtaining good samples for sequencing were very slim for stored samples of over three months in this study.

6. Further studies by veterinary epidemiologists should be done on the tagged doves to evaluate migratory patterns of laughing doves amongst the different residential areas.
7. Veterinary agencies of government should encourage more research on the role of laughing doves in the spread of Newcastle disease to poultry.

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APPENDICES

Appendix I: Biosecurity Assessment of Backyard poultry farms against Newcastledisease.

Columbid ecology studies to assess the potential risk of spread of ENDV to domestic poultry.

Preliminary:

Area: Area Group: Date:

Farm Number: Number of birds: Farm type: GPS location:

Section A. Biosecurity assessment of backyard poultry farms in Zaria.

A.1 Is there a germicide footbath leading into the poultry house? Yes[ ] No [ ]

A.2 Is the poultry house fenced with intact chicken wire that keeps out birds? Yes [ ] No [ ]

A.3. Is there a specific pair of footwear for the pen? Yes [ ] No [ ].

A.4. Are there trees within eyesight? Yes [ ] No [ ]

A.5. Do visitors, egg buyers, poultry buyers, vaccinators enter the poultry house? Yes[ ] No [**]**

A.6. Does poultry feed spill on the ground around the poultry house? Yes [ ] No [ ]

A.7. Are grains, seeds and household flour spread outside in the sun to dry? Yes [ ] No [ ]

A.8. How do you dispose of litter material? a. Bagged up and sealed [ ] b. Spread out to dry c. Kept in a compost bin

A9. Is your poultry feed stored indoors? Yes [ ] No [ ].

A10. Do you own pigeons or doves? Yes [ ] No [ ]

Section B. Assessment of outbreak of Newcastle disease in backyard poultry where doves encroach.

B1. How many times have you suffered outbreaks of Newcastle disease in your backyard poultry flocks?.................

B2. What was the morbidity pattern like? Mild respiratory/enteric disease affecting a few birds [ ] Mild to Severe respiratory disease affecting half the flock [ ] Severe nervous/visceral disease affecting more than half of the flock [ ]

B3. What was the mortality pattern like? A few [ ] Half the flock [ ] Greater than half to all the flock [ ]

B4. What intervention/action was taken during the outbreak? Salvaged sick and medicated /vaccinated the rest of the flock [ ] Salvaged half the birds in the flock [ ] Salvaged all the birds in the flock [ ]

B5. Can you quantify the loss suffered as a result of the outbreak? Minimal [ ] Average [ ] Maximum [ ]

B6. Did you just vaccinate your birds before the outbreak of ND in your flock? Yes [ ] No [ ]

B7. Were other farmers affected by a similar outbreak of ND to that in your flock? Yes [ ] No [ ]

B8. When was the latest outbreak of Newcastle Disease in your farm?........................

B9. What were the major signs of ND exhibited by the birds? Respiratory distress[ ] Enteric signs[ ] Nervous signs [ ]

B10. What lesions were common place in posted dead birds? Bloody trachea [ ]

Bloody gut [ ] Bloody viscera [ ] No lesions [ ]

B11. Were there sudden deaths without apparent signs of illness (died at night)? Yes [ ] No[ ]

Section C: Assessment of management practices that may favour the spread of NDV between domestic poultry and doves/pigeons.

C1. Do you practice the All-In-All-Out policy in stocking and disposing your birds? Yes [ ] No [ ]

C2. Are the premises of the poultry house routinely swept daily and kept clean? Yes [ ] No[ ]

C3. Are poultry hired hands required not to own their own birds or work in other poultry farms? Yes [ ] No [ ]

C4. Is excess waste feed from the poultry house promptly and effectively disposed? Yes [ ] No [ ]

C5. Is poultry feed stored in a safe secure enclosure where doves/pigeons can't access? Yes [ ] No [ ]

C6. Is water for the poultry stored in a secure container where doves/pigeons can't access? Yes [ ] No [ ]

C7. How are carcasses of dead birds disposed in your farm? Incineration [ ], deep burial [ ], and or thrown into the bush [ ]?

C8. Do you keep a farm record book? Yes [ ] No [ ]

Appendix II: Preparation of Dextrose Gelatin Veronal for washing and storage of chicken red blood cells:

Reagents**:**

Reagent	Formula	Quantity
Barbitone sodium	$(C_2H_5)_2C.CO.NH.C.NH.C(ONa).N.CO$	0.3g
Gelatin		0.6g
Calcium chloride	$CaCl_2$	0.02g
Magnesium sulphate	$MgSO_4.7H_2O$	0.12g
Sodium chloride	NaCl	8.5g
D-Glucose	$C_6H_{12}O_6$	10.0g
Distilled water	H_2O	1000ml

Method:

1. The barbitone and gelatin were added to 250 mL of distilled water, and heated over the Bunsen flame till they dissolved completely.
2. The remaining ingredients were added to 600mL of distilled water to dissolve in a second beaker.
3. The two solutions were mixed together in a conical flask.
4. The volume in the conical flask was topped up to 1000mL with distilled water to make 1000mL of DGV.
5. The DGV was transferred into a plastic jerry can, autoclaved at 116^{o}C for 10 minutes, and allowed to cool. The lids were tightened, the jerry can labeled and then stored in the fridge at 4^{o}C till use.

Appendix III: Preparation of pH 7.4 Phosphate Buffered Saline (PBS).

Stock Solutions A and B were prepared as follows.

A: 0.2M solution of monobasic sodium phosphate (31.2g NaH2PO4.2H2O was dissolved in 1000mL of distilled water over the Bunsen flame).

B: 0.2M solution of dibasic sodium phosphate (28.39g of Na2HPO4 was dissolved in 1000mL of distilled water over the Bunsen flame).

To get a PBS with pH 7.4, 19mL of A and 81mL of B were mixed in a conical flask and diluted to a total volume of 200mL. Stock A and B solutions were cooled and kept frozen at -10^{o}C in a deep freezer in separate 1litre jerry cans. PBS pH7.4 was kept in a 1L plastic jerry can and kept in the fridge at 4^{o}C till use.

Appendix IV: Preparation of Viral Transport Medium.

Modifying Naresh's formula for making Stuart's Viral Transport Medium, based on availability of ingredients, the following reagents were used.

Reagent	Quantity
PBS	1,000mL
Cicatrin	500mg
Streptomycin	500mg
Penicillin	6g
Gentamicin	250mg

Method:

1. The ingredients above were dissolved in distilled water prepared under next to aseptic conditions.
2. Using a 2mL plastic Pasteur pipette, 2mL of VTM were transferred into 5mL plain blood tubes with screw caps fitted thereafter.
3. 250 of such 5mL tubes containing VTM were stored frozen in the deep freezer till sample inoculation by swab sticks.

Appendix V: Preparation of 10%, 1% and 0.1% washed Chicken Red Blood Cells**.**

1. 5mL of chicken whole blood was collected aseptically and transferred into EDTA coated blood sample tube.
2. 3mL of the anticoagulant treated chicken blood sample (EDTA whole blood) was decanted into a sterile graduated centrifuge tube.
3. DGV solution was added to the blood in the centrifuge tube to fill it up, the cap screwed on, and then the contents mixed gently.
4. The contents were centrifuged at 500g for 10 minutes.
5. Using a Pasteur pipette, the supernatant was decanted along with a fatty layer of white blood cells and platelets, and a fresh volume of DGV was added to the pellet of packed red blood cells.
6. The contents were mixed, centrifuged at 500g for 10 minutes, and the supernatant decanted for a second wash.
7. To the pellet of packed red blood cells, fresh DGV solution was added again, mixed, and centrifuged at 500g for 10 minutes. Then the supernatant was decanted for a third wash.
8. To the washed chicken red blood cells pellet in the graduated centrifuge tube, DGV was added to make a 10% suspension.
9. From the 10% stock suspension of washed chicken red blood cells, 1 mL was pipetted into a sterile glass tube, and then 9mL of DGV was added to make a 1% suspension of washed chicken red blood cells.

10. From the 1% suspension of washed chicken red blood cells, 1mL was pipetted into another sterile glass tube and 9mL of DGV was added to make a 0.1% suspension of washed chicken red blood cells.
11. The 10% washed chicken RBC suspension was used for the rapid micro-slide HA-HI tests; while the 1% and 0.1% washed chicken RBC suspensions were used for the micro-well titre plate HA-HI tests.

Appendix VI: Detection and Typing of APMV-1 by End-Point RT-PCR and RestrictionEndonuclease Analysis.

Protocol 1

This one-step RT-PCR protocol has been adapted at the Istituto Zooprofilattico Sperimentale delle Venezie (IZSVe) and is based on the primer set describedby Creelan *et al*. (2002). In this protocol, amplification of APMV-1-specific nucleic acid fragments followedby enzymatic digestion (restriction endonuclease analysis, REA) using *Bgl*I was carried out todetect and type strains according to their virulence.

Compared to virus isolation, the relative sensitivity of this protocol for clinical specimenswas 73.44% on a sample basis, rising to up to 91.30% on a case basis (Creelan *et al*., 2002).

Due to the small amplicon size, it is recommended that the RT-PCR results be visualized on silver-stainedSDS-polyacrylamide gels or 2–3% agarose gels.

One-step RT-PCR (AB 9700 thermal cycler) (Super script One step RT-PCR with Platinum Taq, Invitrogen # 10928-042 or –034)

Target: F gene

Sample: 5 µl RNA in a total reagent volume of 45 µl

Primers

Forward NDV-F 4829: 5'-GGTGAGTCTATCCGGARGATACAAG-3'

Reverse NDV-R 5031: 5'-TCATTGGTTGCRGCAATGCTCT-3'

Procedure

Reagenta	Final concentration	1 X Reaction (µl)
RNase-free water	/	13
PCR Buffer 2 _ (MgSO4 2.4 mM; dNTPs 1.6 mM)	1X	50

Primer NDVF 4829	0.2 µM	1.0
Primer NDVR 5031	0.2 µM	1.0
Enzyme mix (RT and Taq)	/	1
Total reagent volume		45

Vortex the mixture for a few seconds.

Aliquot 45 µl into 0.2-ml PCR tubes.

Add RNA		5
Final volume		20

a- Concentration of stock solution

Cycling Conditions

45°C	95°C	95°C	49°C	72°C	72°C	4°C
30 min	2 min	15 s	30 s	30 s	7 min	∞
			(	40 cycles	)	

Detection: Silver-stained SDS-polyacrylamide 7% gel or agarose gel 2%.

Expected amplified fragment: 202 bp.

Restriction endonuclease analysis (**REA**)

Purify the cDNA product using a commercial kit (e.g., High Pure PCR product Purification kit, Roche) following manufacturer'sinstructions.

Digest the PCR amplification product (5 µl in a final volume of 50 µl) with 10 U of *Bgl*I (Roche, Germany; # 621641) and the appropriatebuffer (Buffer H, Roche) for 2.30 h at 37°C.

Visualize the results after gel electrophoresis.

Interpretation of results

Lentogenic APMV-1 restriction pattern: 2 bands of approximately 135 and 67 bp

Meso- and velogenic APMV-1 restriction pattern: 1 band of approximately 202 bp (i.e. no digestion).

Appendix VII: RNA Extraction using BIONEER AccuPrep® Viral RNA Extraction Kit.

1. 200µl of test sample (HA-HI positive oropharyngeal or cloacal swab) was added to 1.5ml micro-centrifuge tubes.

2. 400µl of binding buffer (VB) was added into the tube and mixed by lightly vortexing for 5 seconds.

3. The tubes were incubated for 10 minutes at room temperature.

4. 100µl of isopropanol was added, the tube lightly vortexed for 5 seconds, then spun down for 10 seconds to down the liquid clinging to the walls and the lid of the tube.

5. The binding column was fitted into the 2ml collection tube. The liquid was transferred into the binding column not getting the lid wet.

6. The lids were closed carefully and centrifuged for 1min, at 8,000 rpm.

7. Following centrifugation, the binding column was transferred to a new 2ml collection tube.

8. 500µl of W2 buffer was added, not getting the sides wet; closed the lid, and centrifuged for 1 minute at 8,000rpm.

9. After centrifugation, the binding column was transferred to 2ml collection tube.

10. 500µl of W2 buffer was added, not getting the sides wet; closed the lid, and centrifuged for 1 minute at 8,000rpm. Residual W2 buffer in the column may cause problems in later applications.

11. The collection tube was spun down once more at 13,000 rpm for 1 minute to remove ethanol completely. We made sure there was no droplet hanging from the bottom of the binding column as residual W2 buffer left in the binding column may cause problems in later applications.

12. The binding column was transferred to a 1.5ml collection tube, 50μl of Elution Buffer was added, and allowed to stand for 1 minute to allow the buffer permeate the column.

13. Eluted RNA was retrieved by spinning down at 8,000rpm for 1 minute and used directly for RT-PCR.

Appendix VIII: RBC adsorption-de-adsorption concentration of NDV prior to RNA Extraction and RT-PCR (Jianzhong and Chengqian, 2011).

1. 1ml of pharyngeal or cloacal swab sample in a micro-centrifuge tube was centrifuged for 10 minutes at 12000 rpm twice.
2. 300 µl of supernatant was transferred into a new centrifuge tube and incubated with 50 µl of washed chicken RBC, then spun at 150 rpm for 30 minutes at room temperature to adsorb NDV to RBC. (Adsorption step).
3. The mix was further spun at 750g for 5 minutes at room temperature to concentrate the suspended RBC into a pellet at the base of the tube.
4. Supernatant was decanted.
5. The pellet of RBC with adsorbed NDV was re-suspended in 300 µl of PBS and then 50 µl of 5mM EDTA and 50 µl of beta Mercaptoethanol were added to the mix and incubated at 37^{o}C for 5 minutes. (De-adsorption step).
6. The tube was spun at 1000g for 5 minutes to separate NDV from RBC.
7. Finally, 200 µl of the NDV-rich supernatant was pipetted for RNA extraction, cDNA production and PCR amplification/Agarose Gel Electrophoresis.

Appendix IX: Public Health Action

Feedback Pamphlet from the Veterinary Epidemiology Cross-Sectional Study on **DETECTION OF NEWCASTLE DISEASE VIRUS IN LAUGHING DOVES (*STREPTOPELIA SENEGALENSIS*) AND CHICKENS IN BACKYARD POULTRY FARMS AND THE LIVE BIRD MARKET IN SAMARU- ZARIA, NIGERIA,** sponsored by the MacArthur Foundation Centre for Excellence in Veterinary Epidemiology in partnership with the Department of Veterinary Public Health and Preventive Medicine, Faculty of Veterinary Medicine, A.B.U. Zaria.

Research team: *Dr. Okpanachi J.U., Dr. Kia G.S.N, Dr. Dzikwi A.A., and Prof. Umoh J.U.

Following the successful and careful conduct and completion of the study from samples obtained from free-living laughing doves and backyard poultry; and the assessment of biosecurity measures against Newcastle disease in the following residential areas (Areas BZ, E, G, C, H, Silver Jubilee, Palladan and the Samaru Live Bird Market) it was concluded that:

From this study the following conclusions were made:

1. About 65.7% of the 134 oropharyngeal and cloacal swabs from laughing doves in Samaru-Zaria exhibited haemagglutination 42.1% of which was due to Newcastle disease virus.
2. About 38.1% of the 21 NDV detected in laughing doves in Samaru-Zaria were demonstrated to be lentogenic, 57.1% were mesogenic/velogenic while 4.8% were mixture of both lentogenic and velogenic NDV.
3. Poultry farmers with intact chicken wire barriers that kept out doves were 0.55 times less likely to recall an outbreak of ND than farmers with broken chicken wire barriers.

4. Respondents who reported that doves could not access their poultry feed were 0.69 times less likely to recall an outbreak of ND in their farms when compared to those who reported that doves could access poultry feed.
5. The commonest index of outbreak of ND as recalled by respondents was sudden deaths of chickens overnight.
6. November and August were the months in which respondents recalled the highest outbreaks of ND in their flocks.
7. Backyard poultry farmers who raised over 300 birds and raised layers and broilers together reportedly had the highest outbreaks of ND respectively.
8. Good biosecurity and good management assessment scores resulted in sporadic outbreaks of ND in poultry farms as reported by respondents.

The following recommendations were made:

1. Backyard poultry farmers in the different residential areas should set up community poultry farmers associations to foster and harmonize prevention and control programmes against outbreak of Newcastle disease.
2. Poultry farmers in Samaru should give booster vaccinations against Newcastle disease in the months of June and September so as to confer immunity during the peak periods of outbreaks in the months of August and November.
3. Rainwater collected from rooftops should not be used untreated to water backyard poultry as it may be contaminated with faeces from infected doves shedding NDV.

4. Further studies should be directed towards screening laughing doves for haemagglutinating avian pathogens particularly Avian Influenza virus, Egg-drop syndrome adenovirus, and *Mycoplasma gallisepticum.*
5. For optimum detection of NDV by RT-PCR oropharyngeal and cloacal swabs should be analyzed as soon as they are obtained due to poor cold storage conditions and possible denaturing of viral RNA from incessant power outages in Zaria. Obtaining good samples for sequencing were very slim for stored samples of over three months in this study.
6. Further studies by veterinary epidemiologists should be done on the tagged doves to evaluate migratory patterns of laughing doves amongst the different residential areas.
7. Veterinary agencies of government should encourage more research on the role of laughing doves in the spread of Newcastle disease to poultry.

Thanks for your cooperation.

Together we can kick out Newcastle Disease from Zaria in particular and Nigeria in general!

Veterinary Medicine-Bridging the gap between human and animal health!

Appendix X: Benefactors

Mr. John and Mrs. Catherine MacArthur, founders of the MacArthur Foundation. (Source: www.macfound.org/about/our-history/about-the-macarthurs/)

Appendix XI: National Avian On-Farm Biosecurity Standard – Self Evaluation Checklistfrom the Canadian Food Inspection Agency- Office of Animal Biosecurityused to develop the questionnaire for assessment of biosecurity of backyardpoultry farms in Samaru-Zaria Nigeria,

1. Access Management

    1.1 Designation of zones

        1.1.1 Recognizable zones and access points are in place

        1.1.2 Visual indications are in place to define the Controlled Access Zone (CAZ) and Restricted Access Zone (RAZ)

    1.2 Entry/Movement/Exit

        1.2.1 Workers knowledge on CAZ and RAZ

        1.2.2 Controlled access to CAZ and RAZ plus enforcement

2. Animal Health Management

    2.1 Animal Introduction/Movement/Removal

        2.1.1 Stocking and disposal

        2.1.2 Downtime between stocking flocks

        2.1.3 Stringent additional biosecurity if no all-in-all-out stocking and full downtime observation

    2.2 Ongoing Monitoring of Health Status and Response

        2.2.1 Knowledgeable farm hands

        2.2.2 Daily observation of flocks/culling morbid birds

        2.2.3 Daily mortality log in the flock

        2.2.4 Unusual morbidity/mortality triggers contact with Veterinarian/disease response plan

3. Operational Management

3.1 Mortality and Manure Management

3.1.1 Daily collection and removal of dead birds

3.1.2 Dead poultry storage system

3.1.3 Carcass disposal

3.1.4 Manure handling

3.2 Premises, Building, Equipment and Vehicle Sanitation

3.2.1 Sanitation Program in place

3.3 Facility Maintenance

3.3.1 Facility Maintenance Program in place

3.4 Water/Feed/Bedding Management

3.4.1 Water Management Program in place

3.4.2 Feed source and storage free from contamination

3.4.3 Bedding source and storage free from contamination

3.5 Pest Control Program

3.5.1 Integrated pest control program in place

3.5.2 Garbage effectively and safely disposed

3.6 Biosecurity Program and Training

3.6.1 Knowledge of workers on biosecurity

3.6.2 Review of risks