

**EVALUATION OF REVERSE TRANSCRIPTASE  
LOOP-MEDIATED ISOTHERMAL AMPLIFICATION (RT-LAMP)  
FOR DETECTION OF NEWCASTLE DISEASE VIRUS IN  
NON-TISSUE SAMPLES IN CHICKENS**

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## DECLARATION

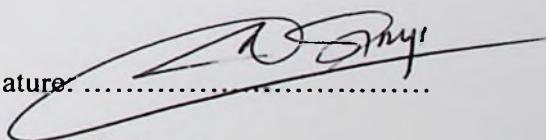
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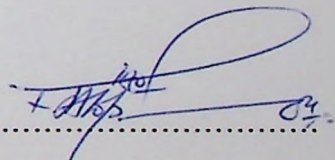
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## DEDICATION

This work is dedicated to my wife Sarah Kafuko Kirunda and my children; Mariam Mutesi Kirunda, Shamirah Mutesi Kirunda and Ibrahim Kirunda for their encouragement, patience and love that enabled me go through this graduate course with perseverance. May the Almighty God continue to bless you!

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## ABBREVIATIONS

|       |                                                        |
|-------|--------------------------------------------------------|
| ACD   | Acid Citrate Dextrose                                  |
| BIP   | Backward Inner Primer                                  |
| B3    | Backward Outer Primer                                  |
| Bst   | <i>Bacillus stearthermophilus</i>                      |
| CAM   | Chorioallantoic Membrane                               |
| cm    | Centimeters                                            |
| DGV   | Dextrose Gelatine Veronal                              |
| DNA   | Deoxyribonucleic acid                                  |
| EDTA  | Ethylene Diamine Tetraacetic Acid                      |
| ELISA | Enzyme Linked Immunosorbent Assay                      |
| FAO   | Food and Agriculture Organisation                      |
| F3    | Forward Outer Primer                                   |
| fg    | Femtogram                                              |
| FIP   | Forward Inner Primer                                   |
| GDP   | Gross Domestic Product                                 |
| HA    | Haemagglutination                                      |
| HI    | Haemagglutination inhibition                           |
| IAEA  | International Atomic Energy Agency                     |
| kbp   | Kilo base pairs                                        |
| LAMP  | Loop-mediated Isothermal Amplification of DNA          |
| LB    | Loop Backward                                          |
| LF    | Loop Forward                                           |
| MAAIF | Ministry of Agriculture, Animal Industry and Fisheries |
| ml    | Milliliters                                            |
| NASBA | Nucleic Acid Sequence Based Amplification              |
| ND    | Newcastle Disease                                      |
| NDV   | Newcastle Disease Virus                                |
| ng    | Nanogram                                               |
| NGDP  | National Gross Domestic Product                        |
| OIE   | Office International des Epizooties                    |

|      |                                   |
|------|-----------------------------------|
| PCR  | Polymerase Chain Reaction         |
| pg   | Picogram                          |
| RNA  | Ribonucleic Acid                  |
| rpm  | Revolutions per minute            |
| SDA  | Strand Displacement Amplification |
| SNP  | Single Nucleotide Polymorphism    |
| TAE  | Tris Acetic acid EDTA             |
| UBOS | Uganda Bureau of Statistics       |
| UV   | Ultraviolet                       |
| v/v  | Volume per volume                 |
| WAHO | World Animal Health Organisation  |
| WHO  | World Health Organisation         |

### ABSTRACT

The ability of Reverse Transcriptase Loop-mediated isothermal amplification of DNA (RT-LAMP) in the amplification of Newcastle disease virus from non-tissue samples was demonstrated. The non-tissue samples used included cloacal and oro-pharyngeal swabs, whose results were compared with those of tracheas (tissue samples).

Samples in this study were obtained from experimental exotic hyssex male chicks on station and growing/adult indigenous chickens in the field. The immunity against Newcastle disease in the experimental chicks was monitored by Haemagglutination inhibition test titres. A total of 138 chicks with very low titres ( $<1\text{Log}_2$ ) were inoculated with the velogenic strain of Newcastle disease virus isolated from embryonated local eggs and among which there were clinical signs including morbidity rate of 100 % and mortality rate of 93.9 % by end of day 7 post infection. Ten naïve hyssex chicks provided the control.

Newcastle disease virus RNA extracted from 41 tracheas, 92 cloacal swabs and 107 oro-pharyngeal swabs collected from experimental chicks immediately at the time of or shortly after death was used in LAMP reaction. Ribonucleic acid from 30 tracheas, 67 cloacal swabs and 59 oro-pharyngeal swabs were collected from clinically sick/dead (naturally infected) grower or adult indigenous chickens from reported outbreaks of Newcastle disease in the field and analyzed by the same method.

In the study, all (100 %) of the samples ( $n=44$ ) that were positive by chromatographic immunoassay were also positive by RT-LAMP method on tracheal samples and cloacal swabs, and 88 % of the oro-pharyngeal swabs collected from the same cases. In addition, 6 of tracheas, 4 of cloacal swabs and 2 of oro-pharyngeal swabs that were negative by chromatographic immunoassay tested positive by RT-LAMP. During the test reactions RT-LAMP amplified RNA from 95.0 % of the tracheal, 78.3 % of the cloacal and 41.1 % of the oro-pharyngeal samples from the chicks. The amplification for field samples was relatively high, with 100 % of the tracheal samples, 97.0 % of the cloacal swabs and 86.4 % of the oro-pharyngeal swabs. In the study the RT-LAMP analytical sensitivity

(detection limit) was 0.85 pg and epidemiological sensitivity and specificity of 89 % and 80 % for cloacal tests and 11 % and 100% of the oro-pharyngeal sample reactions, respectively, for on-station collected samples. Ninety seven per cent and 100 % for cloacal swabs and 80 % and 100 % for the oro-pharyngeal swabs, respectively, were the diagnostic sensitivity and specificity for field diagnosis of Newcastle disease by RT-LAMP. In 75.3 % of the reactions (n=295) conducted by real-time RT-LAMP, amplification was achieved in  $\leq 35$  minutes reaction time with the lowest time of 19:30 minutes. Only test test reactions exceeded 45 minutes. This reaction time was far lower than that taken by nested PCR (6 hours) or real time RT-PCR (1½ hours). Since in this study RT-LAMP has clearly shown a high rate of ND virus detection, it is therefore recommended that the method be adopted for the easier, cheaper and faster diagnosis of Newcastle disease in the field.

## Chapter 1

### General Introduction

#### 1.1 Background

Production of poultry, including chickens, in rural areas is an important agricultural activity of almost all rural communities in Uganda, providing scarce animal protein in the form of meat and eggs as well as being a reliable source of petty cash (MAAIF, 2000). Village chickens also fulfill a number of other functions for which it is difficult to assign any monetary value (Alders *et al.*, 2000). The importance of rural poultry in the national economy of developing countries and its role in improving the nutritional status and income of many smallholder farmers has been very significant (FAO, 2002).

Uganda has an estimated population of 33.0 million poultry 80% of which is reared in the traditional free-range systems in the villages (Mukiibi, 1992; FAO, 2006). Most flocks of village chickens are of small size (8 – 15 birds), mixed age and distributed in many households. However there are a number of challenges to rural poultry production in Uganda (NARO, 1996).

Disease constraints were identified as a limiting factor to improvement of poultry production in Uganda (NARO, 1996), and Newcastle disease (ND) is regarded one of the major diseases of poultry. ND has been identified as the principle factor limiting rural poultry production in all African and Asian countries (Aini *et al.*, 1989; Mukiibi, 1992). However, efficient and cost-effective methods to diagnose the disease in local birds under field conditions are still lacking (NARO, 1996).

Assays including haemagglutination inhibition (HI), virus neutralization, neuraminidase inhibition, haemolysis inhibition, and ELISA have been used to identify Newcastle disease virus (NDV) (Alexander, 1989; Jestin *et al.*, 1989; Wilson *et al.*, 1984). Preliminary diagnosis of ND is based on haemagglutination (HA) and haemagglutination inhibition (HI) tests. According to OIE/VAHO a titre is regarded as positive if there is inhibition at a serum dilution of  $2^4$  or more against 4HA units, or  $2^3$  or more against 8HA Units (OIE, 2000). Other techniques include Oligonucleotide fingerprinting, Isothermal

amplification method, Nucleic Acid Sequence Based Amplification (NASBA) and Sequence Displacement Amplification (SDA) (OIE, 2004). Detection of NDV RNA by PCR was first described by Jestin and Jestin (1991). Several authors have recently used RT-PCR to amplify a fragment of the cleavage site of F-protein and to characterize the resulting DNA fragment by direct sequencing (Jestin *et al.*, 1992; Seal *et al.*, 1995; Collins *et al.*, 1996; Marin *et al.*, 1996; Kant *et al.*, 1997; Yang *et al.*, 1997; Lomniczi *et al.*, 1998) or restriction site analysis (Ballagi-Pordany *et al.*, 1996).

Recently, a novel nucleic acid amplification method termed Loop-mediated isothermal Amplification (LAMP) has been developed (Notomi *et al.*, 2000). The method can amplify a tiny amount of DNA rapidly and specifically under isothermal conditions (Nagamine *et al.*, 2004). The reaction takes place isothermally such that no sophisticated equipment is required. The diagnostic technique is rapid under isothermal conditions, low reaction temperature (63<sup>0</sup>C to 65<sup>0</sup>C), and has high specificity for the target sequence.

## **1.2 Problem Statement**

Newcastle disease (ND) is considered the most important disease affecting chickens, particularly in developing countries including Uganda (Mukiibi, 1992) with morbidity and mortality rates of upto 100 %, especially in most vulnerable chickens. The usual source of infection is assumed to be other chickens. ND epidemics in developing countries result in great socioeconomic losses and affect human health due to reduced food availability (Spadbrow, 1990; Martin, 1992; Alexander, 2003).

In order to prevent further spread of ND virus in cases of outbreak, faster methods of diagnosis are required (Otim *et al.*, 2004). Diagnostic methods, such as Haemagglutination inhibition test, which based on antibody detection, is the most widely used conventional serological method for measuring anti-NDV antibody levels in poultry sera, and considered the standard laboratory test for this disease (Xu, *et al.*, 1997). The method however, does not differentiate between the antibodies due to vaccination or active infection. The test is positive about 4 days post infection.

Diagnostic techniques that can detect the antigen (NDV) in the early days of infection are regarded very important in this case. This is very crucial in the rapid response required in the control of ND. Alternative diagnostic methods, which can be used early in infection and of high sensitivity and specificity such as PCR are labourious, require expensive equipment, are time consuming and require technical skills to perform.

Therefore, diagnostic methods that are fast (obtain results in less than one hour), simple (easy to perform and results easily interpreted), inexpensive (cheaper equipment) and easy (less technical skills required) to use in the diagnosis of ND are required. Recently, Pham and others (2005) developed RT-LAMP method in the diagnosis of ND using tissue samples. This therefore, raised the need to evaluate the use of non-tissue (less invasive) samples in the diagnosis of the disease, since such samples are commonly collected without resentment from farmers in developing countries. The application of sensitivity and specificity concepts as well as measures of agreement beyond chance have to be considered during evaluation of tests so as to develop more refined tests and improved diagnostic ability (Martin *et al.*, 1987).

### **1.3 Specific objectives**

The specific objectives of this study, therefore, were:

1. To evaluate the ability of RT-LAMP assay in the detection of Newcastle disease virus using oro-pharyngeal and cloacal swabs (non-tissue samples) as a basis of diagnosing Newcastle disease.
2. To establish the estimated cost, ease and time (rapidity) implications of using RT-LAMP diagnostic technique in the diagnosis of Newcastle disease, using oro-pharyngeal and cloacal swabs in comparison with RT-PCR.
3. To assess the application of RT-LAMP method in the diagnosis of Newcastle disease in free-range poultry.

### **1.3 Justification**

Poultry production especially that based in the rural areas is an important agricultural activity of almost all rural communities in Africa, providing scarce animal protein in the

form of meat and eggs as well as being a reliable source of petty cash (Alders *et al.*, 2001). Newcastle disease is one of the greatest hindrances to free-range poultry production in such countries.

Newcastle disease occurs worldwide causing significant economic losses in the poultry industry (Alexander, 1988a). The disease (ND) is regarded the principle factor limiting rural poultry production in African and Asian countries. Newcastle Disease may kill up to 80 percent of household poultry in Africa (Aini *et al.*, 1989; Alexander (1997); Bell (1992); Esminger (1992) and Waihenya (2002). In Uganda the disease is endemic (Otim *et al.*, 2004).

Correct diagnosis and treatment are major components of effective disease control and management. Diagnosis is also essential during monitoring the success of treatment. In epidemiological studies, which, essentially, are elements of the preventive strategies of most diseases, correct diagnosis is the main ingredient for the success of such engagements. In the prevention and control of Newcastle disease, early diagnosis is a requirement. Therefore, tests that can detect the causative agent, NDV, early in the disease process are particularly important in the control of spread of the disease.

Field diagnosis of ND in Uganda is currently based mainly on history, clinical presentation and postmortem lesions. Laboratory diagnosis is commonly done by means of Haemagglutination test, virus isolation and Haemagglutination inhibition (HI) test. HI is currently regarded the gold standard in the diagnosis of the disease. However, the diagnosis by HI is based on the principle of developed antibodies, whose earliest time of appearance is about 2-4 days. Various kinds of gene amplification techniques have been developed but commonly have complicated requirements. For example, thermal cycling is required for PCR. Isothermal amplification method, Nucleic Acid Sequence Based Amplification (NASBA) and Sequence Displacement Amplification (SDA) requires multiple numbers of the enzyme and specific reagents. More convenient and rapid methods for the diagnosis of diseases are therefore desired (IAEA/FAO, 2006).

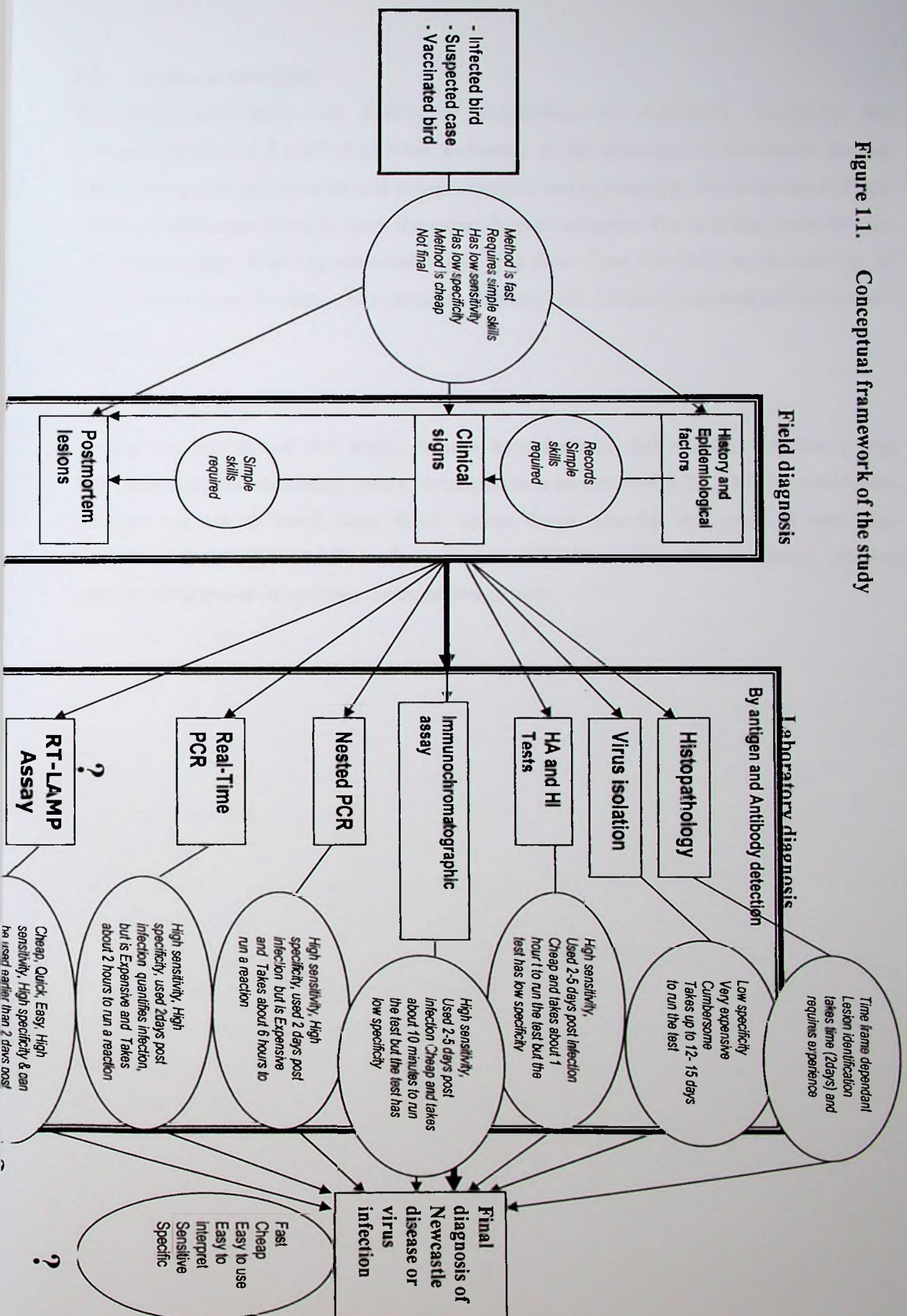
A diagnostic technique, such as LAMP assay, that is based on detection of antigen may prove to be an extremely vital tool in the early detection of infection and hence faster diagnosis and/or prevention of spread of Newcastle disease or its control. The evaluation of various options of use of LAMP assay, a novel isothermal amplification method, which only requires a single DNA polymerase and that is capable of amplifying DNA under isothermal conditions with high sensitivity and specificity (Notomi *et al.*, 2000), in the diagnosis of infectious diseases including Newcastle disease, is therefore necessary.

The purpose of this study, therefore, was to contribute towards effective control of Newcastle disease in free-range chickens through easy, rapid and cheaper diagnosis of NDV infection by evaluating RT-LAMP using less invasive samples.

### **1.5 Significance of the study**

The findings of the study are expected to assist in the choice of an alternative method for the rapid and cheaper diagnosis of Newcastle disease in chickens in the Ugandan environment using less invasive samples (swabs) from the cloaca and oro-pharynx.

Figure 1.1. Conceptual framework of the study



## **1.6 Scope of the study**

This study specifically was limited to establishing the sensitivity, specificity and precision of the RT-LAMP diagnostic technique in the detection of Newcastle disease virus from non-tissue (cloacal and oro-pharyngeal swabs) samples as compared with the results of the tissue samples from the same disease subjects. The test employed freshly collected samples from experimental chicks and those from the field in sub-counties of Iganga and Tororo districts – representing districts with frequent outbreaks of Newcastle disease.

## **1.7 Hypotheses**

During the conduct of this study, it was hypothesized that the test sensitivity and specificity, and reaction time, ease of interpretation and cost of RT-LAMP on non-tissue samples are not different from those where tissue samples are used. It was also hypothesized that RT-LAMP analysis can identify Newcastle disease among clinical cases of chickens on free-range management system.

## Chapter 2

### 2.0

### Literature Review

#### 2.1 Newcastle disease

The first documented evidence of Newcastle Disease (ND) in Uganda occurred in 1955 although there had been earlier reports of a similar disease in other parts of the country (Mukiibi, 1992). The disease is caused by the Newcastle disease virus (NDV), an enveloped RNA virus of the paramyxoviridae family in the order of mononegavirales (Murphy *et al*, 1997). The paramyxovirus genus consists of avian paramyxoviruses (APMV) serotypes 1-9. NDV is the only member of APMV- 1 serotype. The Newcastle Disease virus (NDV) strains have been classified into three strains: less virulent or lentogenic, moderately virulent mesogenic and the highly virulent or velogenic strain (Alexander and Allan, 1974). Based on the clinical signs induced in infected chickens, the virus is classified into 5 pathotypes (Beard and Hanson, 1984).

Although NDV is inactivated at a temperature of 55°C for five minutes or by direct sunlight for 30 minutes, its infectivity remains unchanged at 4°C for a few days, at -20°C for several weeks, and at -70°C for months or even years (Forlich *et al*, 1982). The virus is known to survive in fresh eggs for several years when frozen. In un-plucked carcasses stored at 4°C, virus persists on the skin and in bone marrow for 4-6 months (Omojola and Hanson, 1986). Virus in the faeces can survive for more than 8 weeks in hot dry tropical areas at the temperature of 40°C (Werner, 1989).

During transmission virus excretion commences before clinical signs of ND occur and may be from birds with the clinical disease, vaccinated birds, latently infected birds and survivors of natural infection which still harbour the agent, which may even act as reservoirs. Transfer of the virus between chickens is by virus-bearing airborne droplets that are generated in the respiratory tract of the infected chickens for a period of over 2 to 4 days. Virus is also released in the faeces, which contaminate the litter, feed, and water (Alexander, 1988b).

Scavenging, as done by rural chickens, exposes them to a greater risk of getting in contact with wild birds that are carriers of the NDV than intensively kept chickens (Awan *et al.*, 1994). This implies that chickens in a village get infected at different times producing a near cyclic pattern of the disease. In their serological study in Uganda, Mukiibi and Olaho-Mukani (1998) found that the birds from one village out of three sampled did not have antibodies to NDV, which indicates that even in enzootic areas, chickens in some villages though can remain fully susceptible to infection with virulent NDV.

Under industrial production conditions, ND has been successfully controlled through vaccination. A similar concept for the control of ND in free-range poultry production has been developed but the disease still remains the single greatest constraint to rural poultry production throughout the developing world (Awan *et al.*, 1994). Likely factors involved might include vaccination failure due to exposure to immunosuppressive agents, other intercurrent infections and vaccine potency.

## **2.2 Diagnosis of Newcastle disease**

Diagnosis involves the use of clinical history, clinical signs of sick birds, gross pathology and laboratory tests. In the laboratory the antigen (NDV) or antibodies against NDV infection are targeted by the various diagnostic techniques in use.

### **2.2.1 Clinical signs of Newcastle disease**

The incubation period of ND after natural exposure varies from 2-15 days (5-6 days on average). The appearance of the clinical signs and the severity of ND depend on strain of the virus, host species, age, immune status, co-infection with other organisms, environmental stress, social route of exposure and the virus dose (McFerran *et al.*, 1988). NDV strains and their isolates have the ability to produce distinct signs and severity, which vary even within the same host. None of the clinical signs or pathological lesions may, therefore, be regarded as pathognomonic for ND (Alexander, 1997).

With extremely virulent viruses, the disease may appear suddenly, with high mortality occurring in the absence of other clinical signs. In outbreaks in chickens due to the velogenic virus ND pathotype, clinical signs often begin with listlessness, increased respiration, and weakness, ending with prostration and death. There may be oedema around the eyes and head. Green diarrhea is frequently seen in birds that do not die early in infection, and prior to death, muscular tremours, torticollis, paralysis of legs and wings, opisthotonos may be apparent. Mortality frequently reaches 100 % in flocks of fully susceptible chickens (McFerran, 1988) and a marked or drastic fall in egg production.

The clinical signs produced by specific viruses in other hosts may differ widely from those seen in chickens. In general, turkeys are as susceptible as chickens to infection with NDV, but clinical signs are usually less severe (McFerran, 1988). Although ducks and geese are usually regarded resistant to NDV infections even to strains most virulent for chicken, outbreaks of severe disease in ducks infected with NDV have been described (Higgins, 1971). Outbreaks of virulent ND have been reported in most game bird species (Roy *et al*, 2004) and the disease appears similar to that in chickens.

The capacity of a strain to bring about immunity to disease, irrespective of the actual serological changes, depends on the amount of the virus birds are exposed to, the route of application and the virulence of the strain. Thus, ND infections induced by virulent strains are more immunogenic among survivors than those caused by less virulent strains. However, immunity is not directly related to the presence of circulating antibodies, and birds, which no longer possess detectable amounts of haemagglutination-inhibiting or neutralizing antibodies, may, nevertheless, be able to withstand exposure to virulent virus (Alexander, 1990).

### **2.2.2 Gross pathology of Newcastle disease**

Lesions in the gastrointestinal tract are oedematous, haemorrhagic, necrotic and ulcerative. There is marked oedema of the interstitial tissues of the neck. Haemorrhages occur in the trachea, corresponding to the rings of cartilage, and in the proventriculus,

gizzard, peyer's patches, caecal tonsils and other aggregations of the lymphoid tissues in the intestinal wall. Small flat, red or purple (petechial) haemorrhages may be seen on the breast muscle, heart muscles, peritoneal adipose tissue and serosal lining (Geering and Forman, 1995; Calnek *et al.*, 1997).

### **2.2.3 Laboratory diagnosis of Newcastle disease**

Samples for ND diagnosis can be taken from live or recently dead birds. Swabs of oropharyngeal and cloacal contents or intestine contents, brain, heart, blood, lungs, air sacs, intestine, spleen, kidney and liver should be collected aseptically and processed, either separately or as a pool, for conformational and differential diagnostic purposes (Calnek, 1997).

Laboratory diagnosis of ND is done through serological tests, establishment of biological properties of virus strains and molecular techniques (Calnek, 1997; Alexander, 1989; Alexander, 1997; Kant *et al.*, 1997). The assays that have been used to identify NDV include Haemagglutination test and Haemagglutination inhibition (HI) test, virus neutralization, neuraminidase inhibition, haemolysis inhibition, and ELISA in domestic birds (Alexander, 1989; Jestin *et al.*, 1989; Wilson *et al.*, 1984). HI test is the most widely used test (OIE, 2004). Among the common antigen-based assays are RT-PCR and Nested PCR. Another recently developed test for rapid diagnosis of ND is chromatographic immunoassay (<http://www.anigen.co.kr>, 2005).

Diagnosis of ND at the preliminary stage is based on haemagglutination (HA) and haemagglutination Inhibition (HI) tests. Using HI test, a sample may be regarded as positive when there is inhibition at a serum dilution of  $2^4$  or more against 4HA units, or  $2^3$  or more against 8HA Units (OIE, 2000).

#### **2.2.3.1 Loop-mediated isothermal amplification (LAMP)**

Recently a novel nucleic acid amplification method termed Loop-mediated isothermal Amplification (LAMP) was developed (Notomi *et al.*, 2000). This method relies on autocycling strand displacement DNA synthesis performed using the Bst DNA

polymerase large fragment (Mori *et al.* 2001; Nagamine *et al.*, 2002; Notomi *et al.*, 2000). The method can amplify a tiny amount of DNA rapidly and specifically under isothermal conditions (Nagamine *et al.*, 2004). In this method DNA synthesis is initiated with 2 inner primers, 2 outer primers and DNA polymerase with strand displacement activity. The inner primer contains the complementary sequence to the elongation strand sequence of the target gene. The outer primer is designed for the synthesis of the starting structure of LAMP cycling. The reaction takes place isothermally so that no sophisticated equipment (such as thermal cycler) is required. Furthermore, no denaturing process is required for amplification reaction. The gene amplification is confirmed in one hour with the template DNA of merely 6 copies. The product consists of the alternately inverted repeat of sequence of the target. The products are obtained as the result of LAMP reaction. In LAMP reaction, the amount of the synthesized DNA is large enough to make the detection system simple. LAMP method is, therefore, useful for the rapid gene detection and diagnosis (Nagamine *et al.*, 2002).

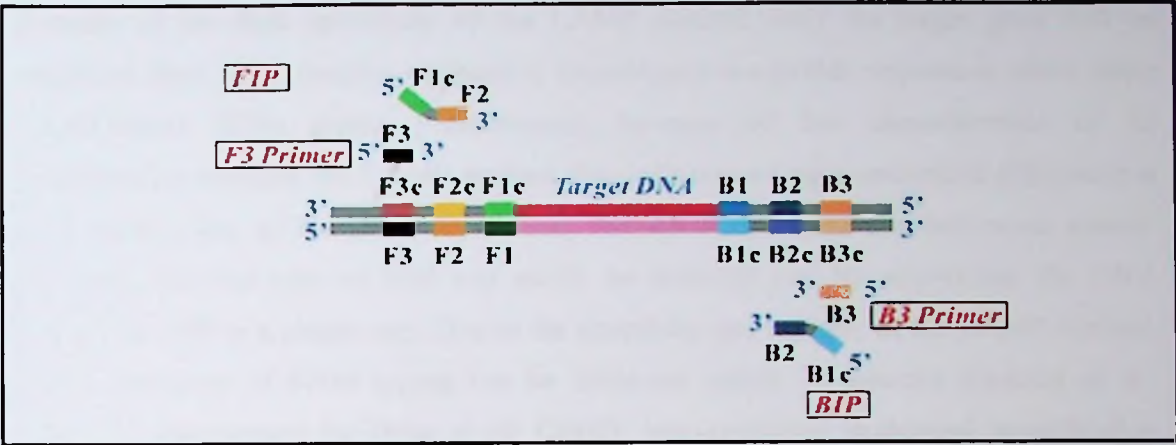
#### **2.2.3.2 Characteristics of LAMP**

The characteristics of LAMP are rapidity under isothermal conditions, low reaction temperature (63°C to 65°C), and high specificity for the target sequence. The technique provides high amplification efficiency, with DNA being amplified  $10^9 - 10^{10}$  times in 15 – 60 minutes. This is attributable to recognition of the target sequence at six independent sites in the initial stage and at four independent sites during the last stages. In addition, it requires only four primers, a DNA polymerase, and a regular laboratory water bath or heat block for reaction, making it simple to perform (Notomi *et al.*, 2000 and Mori *et al.*, 2001). LAMP method requires no special reagents; no sophisticated temperature control device required; the template can be simply detected through the presence of amplified product. Since it only requires simple equipment, cost effective genetic test can be achieved.

#### **2.2.3.3 LAMP primer design**

The LAMP primers are designed using primer explorer software available at the Eiken Chemical Co. Ltd. Website (<http://primerexplorer.jp/e/>). As shown in Figure 2, the 4

types of primers are designed based on 6 distinct regions of the target gene; the F3c, F2c and F1c regions at the 3' side, and B1, B2 and B3 regions at the 5' side.



**Figure 2.1. Schematic illustration of Primer Design showing four primers and their positions on the target DNA.** Source: Eiken Chemical Co. Ltd. (<http://loopamp.eiken.co.jp/e/lamp/primer.html>)

Forward Inner Primer (FIP) consists of the F2 region at the 5' end. F3 Primer, Forward Outer Primer consists of the F3 region that is complementary to the F3c region. Backward Inner Primer (BIP) consists of the B2 region (at the 3' end) that is complementary to the B2c region, and the same sequence as the B1c region at the 5' end. And Backward Outer Primer (B3 Primer) consists of the B3 region that is complementary to the B3c region.

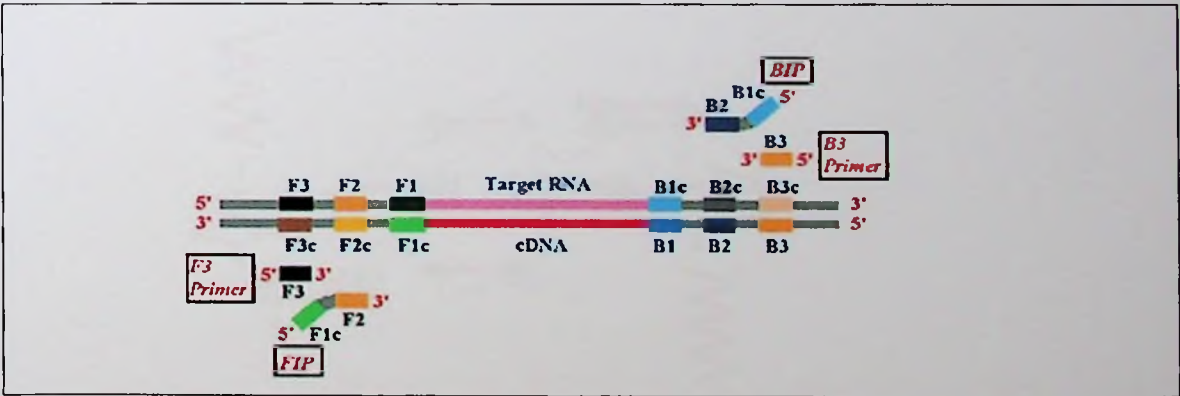
**2.2.3.4 Mechanism of LAMP reaction**

During the start of the reaction, as and when the double stranded DNA is in the condition of dynamic equilibrium at the temperature around 65°C, one of the LAMP primers can anneal to the complimentary sequence of double stranded target DNA, and then initiates DNA synthesis using the DNA polymerase with strand displacement activity, displacing and releasing a single stranded DNA. With the LAMP method unlike with PCR, there is no need for heat denaturation of the double stranded DNA into a single strand. Through the activity of DNA polymerase with strand displacement activity, a DNA strand complementary to the template DNA is synthesized, starting from the 3' end of the F2

region of the FIP (Notomi *et al.*, 2000). Notomi *et al.* (2000) described the entire principles involved in the mechanism of the amplification reaction of LAMP assay. Because of the high specificity of the LAMP method, only the target gene will be amplified from gene samples containing homologous nucleotide sequences when using LAMP-based SNPs typing. Furthermore, because of the characteristics of its amplification reaction, the LAMP method discriminates a single nucleotide difference at each cycling step of the DNA replication, through both “sense and anti-sense strand” reactions, and the type of SNP can easily be detected just by amplifying the DNA containing SNP in a single step. Due to the simplicity and rapidity of the LAMP method, simple detection of SNPs typing can be achieved within 30 minutes (Notomi *et al.*, 2000). In experiments by Baba *et al.* (2002), loop-mediated isothermal amplification (LAMP) was adopted when it was realized that LAMP, not only avoided the thermal cycling, but also offered higher specific amplification than traditional PCR.

### 2.2.3.5 Detection of LAMP products

After the amplification, the products could be either analyzed by the integrated microchip electrophoresis or directly observed by the naked eyes by adding some fluorescent dye like SYBR Green 1. Both simple detection and real-time detection of the reaction are possible.



**Figure 2.2. Schematic illustration of Reverse Transcriptase Primer Design showing four primers and their positions on the target DNA.** Source: Eiken Chemical Co. Ltd. (<http://loopamp.eiken.co.jp/e/lamp/primer.html>).

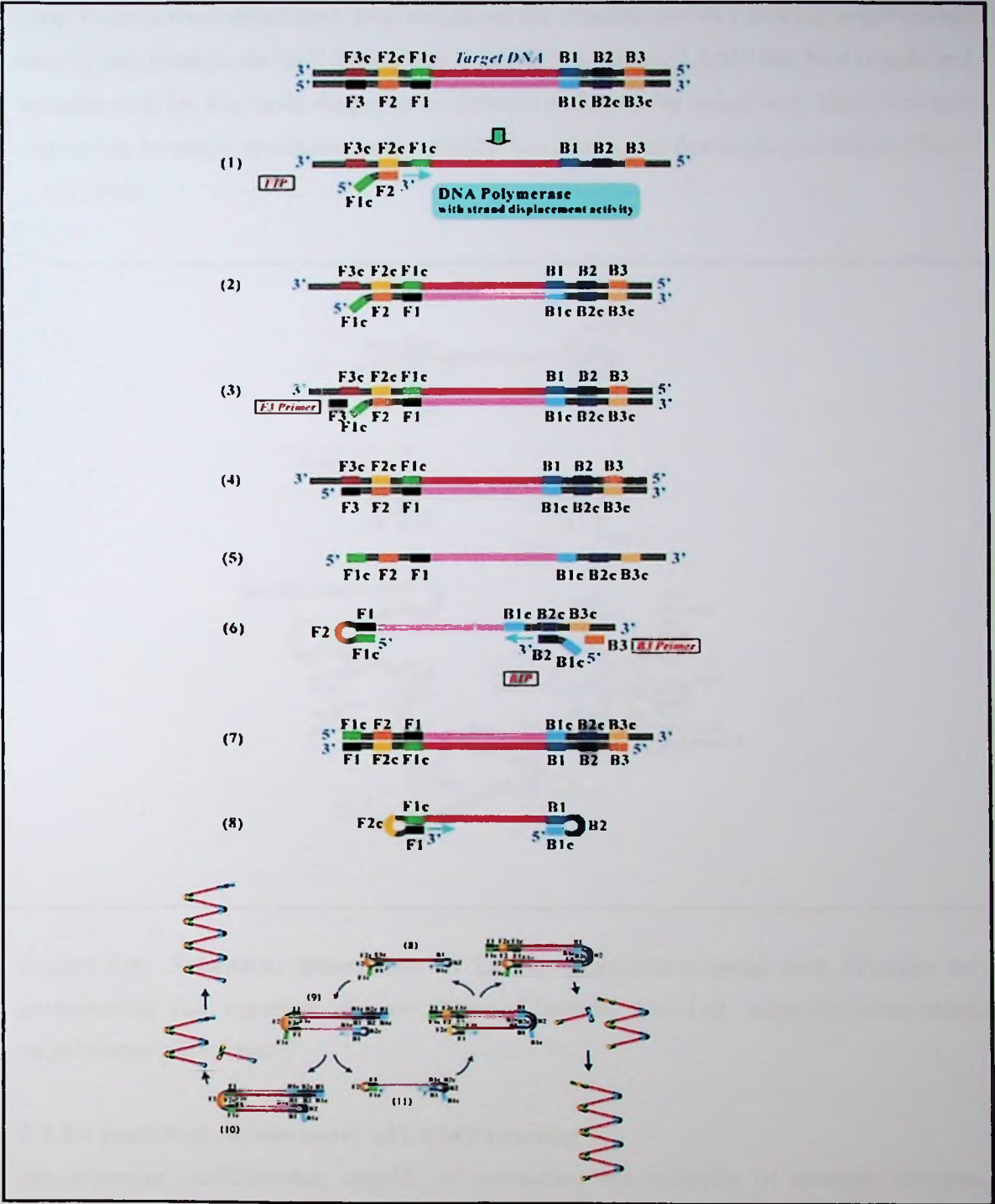
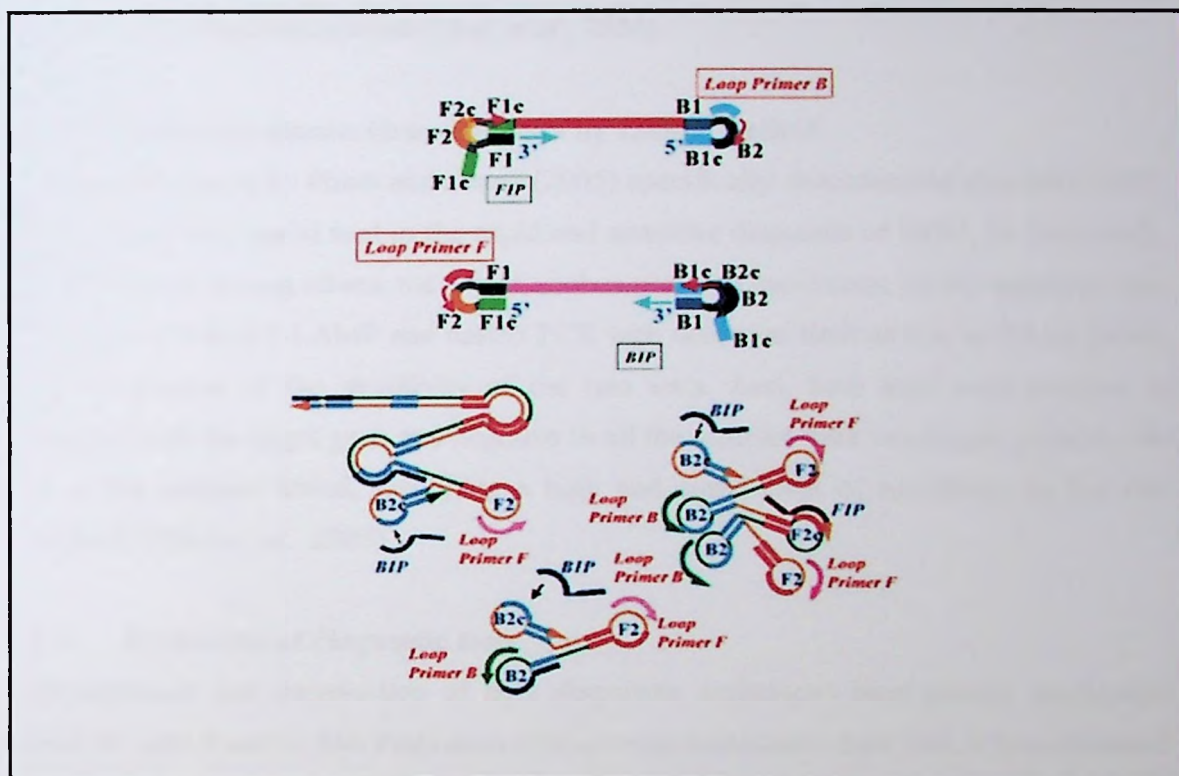


Figure 2.3. Schematic illustration of LAMP mechanism. Source: Nagamine *et al* (2002).

Loop Primers were developed, they accelerate the reaction and can shorten amplification time by one third to one half (Nagamine *et al.*, 2000). Hence, LAMP can be a simple and valuable tool for the rapid diagnosis of infectious disease by using very basic facilities and should be easily applicable in the clinical laboratories of developing countries (Pham *et al.*, 2005).



**Figure 2.4. Schematic illustration of LAMP mechanism using loop primers for accelerating the reaction.** Source: Eiken Chemical Co. Ltd. (<http://loopamp.eiken.co.jp/e/lamp/primer.html>)

#### 2.2.3.6 Real-time turbidimetry of LAMP reaction

An apparatus, turbidimeter, capable of measuring the turbidity of multiple samples simultaneously while maintaining constant temperature to conduct real-time measurements of the changes in the turbidity of LAMP reaction was developed. The turbidimeter is designed to detect only the by-product of the DNA synthetic reaction, the

pyrophosphate ions as the insoluble magnesium pyrophosphate. The relationship between the amount of amplified DNA and the turbidity is measured by the real-time turbidimeter. The time required for the turbidity of the LAMP reaction solution to exceed a given value was observed to depend on the amount of initial template DNA. Real-time measurement can be achieved, thereby permitting quantitative analysis of minute amounts of nucleic acid present in the sample with high precision, and graph of the amplification reactions plotted (Mori *et al.*, 2004).

#### **2.2.3.7 Newcastle disease virus detection by LAMP method**

Results of a study by Pham and others (2005) specifically demonstrated that the LAMP-based assay is a useful tool in the rapid and sensitive diagnosis of NDV. In their study, that involved, among others, the use of trachea and lung specimens, all the samples, were positive by both RT-LAMP and nested PCR with detection limit as low as 0.5 pg during the comparison of the sensitivity of the two tests. And, both tests were positive in samples with the target gene and negative in all the samples with non-target genes in 100 % of the samples tested, exhibiting a high and equal level of specificity by the two methods (Pham *et al.*, 2005).

### **2.3 Evaluation of diagnostic tests**

Development and introduction of new diagnostic techniques have greatly accelerated over the past decades. The evaluation of diagnostic techniques, however, is less advanced than that of drug treatments. Unlike with drugs, there are generally no formal requirements for adoption of diagnostic tests in routine care. In spite of important contributions by Feinstein (1985) and Sackett (1985), The methodology of diagnostic research is poorly defined compared with study designs on treatment effectiveness, or on aetiology. Therefore, it is not surprising that methodological flaws are common in diagnostic studies (Sheps and Schechter, 1984; Lijmer *et al.*, 1999).

Under usual circumstances, when a new test for disease is developed, its results are often compared to those from the existing standard, yet imperfect test (gold standard). This may be done for the purpose of measuring the level of agreement (Martin *et al.*, 1987). If a gold standard is not available, it is only possible to assess the agreement between

different tests, without assuming that one test is the best. The logic of using the approach is that agreement between tests is evidence of validity, whereas disagreement suggests that the tests are untrustworthy (Thrusfield, 2001). Therefore, in circumstances the sensitivity and specificity of a test to be evaluated is required and yet such a test is biologically similar to the standard test(s), its results can be compared with those of such a test(s) in order to determine the two properties for the new test (Martin *et al.*, 1987). For this purpose, animals positive to all tests in the test bank are assumed diseased and animals that are negative to all the tests in the bank assumed to be disease-free (Martin *et al.*, 1987; Mateu-de-Antonio *et al.*, 1993). However, in such a case the results of the comparisons should be prefixed with 'relative', to indicate that the determinations were based on biologically related tests (Martin *et al.*, 1987).

There are a number of ways to evaluate a diagnostic test, varying from technical studies of calibration to controlled trials of the effect upon patient outcome (Martin *et al.*, 1987). However, evaluation of a diagnostic test usually refers to measurement of the diagnostic parameters of the test: sensitivity, specificity, positive and negative predictive values, and likelihood ratios (Martin *et al.*, 1987).

The sensitivity of a diagnostic method is the proportion of true positive that are detected by the method while the specificity of the method on the other hand, is the proportion of true negatives that are detected. Sensitivity and specificity describe the discriminatory power of a test based on single biologic sample taken at a point in time (Martin *et al.*, 1987). In the immunologic, pharmacologic and analytical usage, sensitivity of a test refers to the ability of the test to detect a small amount of, among others, the antibody and antigen (Martin *et al.*, 1987). In analytical sensitivity, the more sensitive the test, the smaller the amount of antigen (for example) it will detect (Thrusfield, 2001). The sensitivity and specificity of a diagnostic test are important in deciding the value of the test in disease control campaigns and in categorizing animals in observational studies (Thrusfield, 2001). The sensitivity as a parameter of validity of a diagnostic test is termed diagnostic sensitivity (Stites and Rodgers, 1991). Sensitivity and specificity can be quoted either as a probability between 0 and 1, or as a percentage (Thrusfield, 2001). Sensitivity and specificity can be calculated for tests in which the variable that is

measured is nominal or dichotomous, for example, presence or absence of antigen (Thrusfield, 2001). Once a diagnostic technique measures what it purports to measure, it is valid (Thrusfield, 2001). Generally, at sensitivity and specificity of 99 % and 99 %, respectively, a test can be regarded relatively good for epidemiological studies (Martin *et al.*, 1987). For laboratory confirmatory purposes in disease diagnosis, a factor required in disease eradication campaigns, the required characteristic of a test is the specificity that is as high as 99.9 % (Martin *et al.*, 1987).

## Chapter 3

### 3.0 General Materials and Methods

#### 3.1 Sample collection

Various types of samples were collected from chickens during the study. Tracheas, lungs and spleens were first collected from the field for ND virus isolation. Similarly, blood (for serum) was collected for HI test, cloacal swabs for chromatographic immunoassay (*Anigen test*) and tracheas, cloacal and oro-pharyngeal swabs for RT-LAMP analysis.

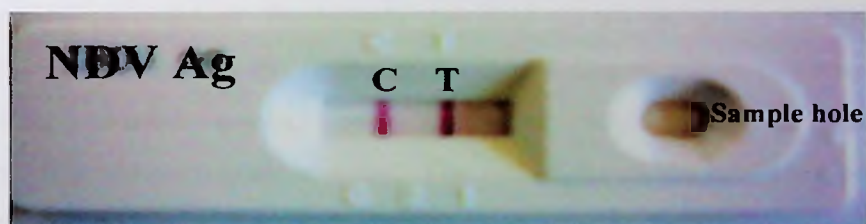
The chicken tracheal tissues and the non-tissue samples were collected from birds immediately after death. For examination and samples collection from live chickens, the examination and sample collection procedures described by Hofstad (1984) and Fowler (1996) were used. Similar procedures described by the same authors were used during collection of tracheal tissues at postmortem. A thorough postmortem was done before sample collection. Postmortem was done purposely to verify that pathological lesions observed were typical for NDV infection or exposure (Calnek *et al.*, 1997).

#### 3.2 Procedures used in chromatographic immunoassay

A qualitative detection of Newcastle disease virus antigen was done during the study for the purpose of confirming ND outbreak reports and during confirmation of specific cases. The method used for this was chromatographic immunoassay. The Newcastle disease virus antigen was detected in avian cloacal swabs. This test uses specially selected Newcastle disease virus antibodies that are used in the test band as both capture and detector materials. *Anigen Rapid NDV Ag Test Kit*<sup>®</sup> (Animal Genetics, Inc. Wonheon, Yeongtong, Suwon, Kyonggi-do, Korea), a quick diagnostic tool, was specifically used to confirm Newcastle disease virus infection/exposure (using cloacal swabs) before sample collection from a given flock during the study. Diagnosis of ND by this test was reported to have sensitivity and specificity of 94.7 % and 96.4 %, respectively, when it was compared with Reverse Transcriptase Polymerase Chain Reaction (RT-PCR). Also reported is its detection limit of 0.25 Haemagglutination Units (<http://www.anigen.co.kr>, 2005). The test was used as a gold standard in some part of this study. The test

procedures, as described by the manufacturer, were followed in the application of this diagnostic technique.

Cloacal swabs of 10 chicks or adult chickens from those clinically sick in a flock were taken and immediately used for the detection of Newcastle disease virus. In this test procedure, samples were collected from the cloaca using the swabs provided with *Anigen Rapid NDV Ag Test Kit*<sup>®</sup>. After swabbing, the cloacal swab was inserted into the specimen tube containing one millilitre of assay diluents and thoroughly agitated in the diluent to extract the virus. The immunochromatographic assay test strip was removed from the foil pouch and placed on a flat and dry surface. Using the disposable dropper provided, four (4) drops of the sample extract from the tube were added into the sample hole (Figure 3.1). Slowly, drop by drop. As the reaction began taking place, the colour (expected to be purple) was watched across the test window into the centre of the test device. If no migration appeared to have taken place after 1 minute, one more drop of the sample extract was added to the sample hole. Test results were interpreted at 5 – 10 minutes. No decision was made after 20 minutes of the test reaction. Only a test result indicating the presence of two colour bands ('T' and 'C') within the result window of the test device, no matter which band appeared first, was taken to indicate a positive result (Figure 3.1).



**Figure 3.1. Positive chromatographic immunoassay (Anigen test)**

C – Control result and T – Test sample result

### **3.3 Sample processing and preservation**

Samples (tracheal tissues, cloacal swabs and oro-pharyngeal swabs) were, immediately after collection, preserved in 50 % glycerol. Aliquots of allantoic fluid and samples

preserved with glycerol were immediately kept at -20°C. Field samples were transported preserved on dry ice before refrigeration at similar temperature.

Prior to lab testing, frozen samples were left on the bench overnight to allow for thawing before being subjected to further processing or analysis. Tracheal tissue samples were first minced using sand and the supernatant aliquoted. All categories of samples were vortexed to obtain a homogenate.

### **3.4 Sample size determination**

The need for sample size adequacy to provide the desired information with sufficient precision is often ignored in diagnostic studies. Progress in diagnostic performance often consists of a series of small steps that gradually increase certainty rather than by one big breakthrough. Evaluating small steps, however, requires large study populations (Knottnerus *et al.*, 2002).

A review of 8,999 articles (43 of which were on non-screening tests) on diagnostic accuracy from eight journals, on studies of determination of specific test's sensitivity and specificity by Bachmann and others (2006) generally revealed that the interquartile range of the sample size for such studies was 71 – 350, and the median sample size was 117. For the successful running of a McNemar's statistical test at least 20 matched-pair sample results are required. The sample size used for the on-station study was 240 and 156 was the sample size for the field part. Of the 92 cloacal swabs from on-station and 67 of the field cloacal swabs, only 30 and 44, respectively, were analysed by immunochromatographic assay. The rest of the swabs could not be analysed due to lack of enough test reagents (test strips).

### **3.5 Sampling method used during sample collection**

Samples intended for LAMP assay were collected only from chickens in the clinical state of Newcastle disease or immediately after death. Tracheal tissue samples were collected after postmortem examination. Only carcasses with ND postmortem presumptive lesions such as haemorrhages in the trachea and proventriculus and petechial haemorrhages in the heart muscles were considered for trachea collection. The samples were immediately

labeled based on the study name (L for LAMP), tag number and organ from which each was collected.

### **3.6 Procedures used during RNA extraction from samples ([www.quagen.com](http://www.quagen.com), 2005)**

Ribonucleic Acid (RNA) from the tracheal tissues and cloacal and oro-pharyngeal swabs was extracted by use of the QUAamp® Viral RNA Mini Kit. Strictly procedures of the manufacturer of QUAamp® Viral RNA Mini Kit (Quagen Inc., Valencia, CA, USA), as described in the kit handbook, were followed during RNA extraction. The samples and the elution buffer from the test extraction kit were always first equilibrated to (thawed at) room temperature (20°C) before starting the extraction process. The two buffers used in the precipitation of RNA and the Carrier RNA supplied with the kit were checked for correct preparation before use. The precipitates in the lysis buffer and Carrier RNA, provided in the kit, were first redissolved, by heating and cooled to room temperature before use. Only RNAase free tubes and cryo vials (Ambion, Applied Biosystems, Woodward, USA) were used during the RNA extraction process and storage of the eluted RNA, respectively. All centrifugation steps during the extraction process were done at room temperature.

All samples to be extracted were first checked for their unique identification numbers and these were recorded accordingly. The volume of samples used for extraction for all samples was 140 µl. Each sample was given a laboratory identification number to correspond to sample record in data base, which was used to label the cryo vials for storing the eluted RNA from each specific sample.

Each time a sample was extracted, a volume equivalent to 560 µl of the prepared lysis buffer containing Carrier RNA was pipetted into a 1.5-ml microcentrifuge tube. The sample was first mixed by pulse-vortexing for one minute in order to free cells from the swab and/or thoroughly mix the cells. Immediately after vortexing 140 µl was drawn from the bottle and added to the lysis buffer/Carrier RNA in the centrifuge tube. These were then mixed by pulse-vortexing for 15 seconds. This was, again done to ensure efficient lysis and yielding of a homogeneous solution. In order to ensure complete viral

particle lysis, the sample/buffer mixture was then incubated at room temperature (20°C) for 10 minutes followed by centrifugation at 6,000 x g for one minute in order to remove drops from the inside of the lid of the tube. Ethanol (99.7 %) amounting to 560 µl was added to the sample solution, mixed by pulse-vortexing for 15 seconds and briefly centrifuged 1000 rpm for one minute to remove any drops from inside the lid of the microcentrifuge tube. This was done in order to ensure that there was efficient binding of RNA to ethanol and that, a homogeneous solution was yielded. Carefully 630 µl of the solution (from the previous step) was applied to a QUAamp spin column (in a 2-ml collection tube) without wetting the rim. The cap was closed, and then centrifuged at 6,000 x g for one minute. A QUAamp spin column was then placed into a clean 2-ml collection tube, and the tube containing the filtrate discarded. During centrifugation each spin column was properly closed in order to avoid cross-contamination. A QUAamp spin column was carefully opened, and step before this one, repeated. A QUAamp spin column was again carefully opened, and 500 µl of the buffer to again wash away the alcohol from the RNA was added. The cap was closed, and spin column centrifuged at 6,000 x g for one minute. The spin column was then placed in a clean 2-ml collection tube, and the tube containing the filtrate discarded. The column was, at this stage, carefully opened, and 500 µl of another type of wash buffer added to further clean the RNA. This was accompanied with closure of the cap and centrifugation at full speed (20,000 x g) for three minutes. The column was finally placed in a clean 1.5-ml microcentrifuge tube and the collection tube initially containing the filtrate discarded. A QUAamp spin column was carefully opened and 60 µl of elution buffer, equilibrated to room temperature, added, the cap closed, incubated at room temperature for one minute and centrifuged at 6,000 x g for one minute.

The eluted RNA was first stored at -80°C but later precipitated to facilitate shipment. Samples were shipped to Obihiro University in Japan where analysis was done.

The working bench was disinfected each morning before sample preparation. Only powder free latex gloves were used during the RNA extraction processes. Gloves were changed after processing of 10 samples during the extraction process. Pipette adaptors were constantly wiped with 70 % alcohol or JIK during the entire process of extracting

RNA from each batch of samples. In order to avoid cross contamination during the extraction process pipette tips with aerosol barriers (filters) (Ambion, Applied Biosystems, Woodward, USA) were used. Pipette tips were changed between samples during the entire extraction procedure when each batch of samples was processed.

The precipitation involved the use of ethanol precipitation of nucleic acids procedures ([http://openwetware.org/wiki/Ethanol\\_precipitation\\_of\\_nucleic\\_acids](http://openwetware.org/wiki/Ethanol_precipitation_of_nucleic_acids)). The materials used included absolute ethanol (99.7 % ethanol), sodium acetate and refrigerated centrifuge. During the procedure, the RNA samples were taken from the freezer (-80°C) on to a working bench. To each sample was added absolute ethanol of twice the volume of the sample. Sodium acetate (of pH 5.2) at a rate of  $\frac{1}{10}$  the volume of the sample was added to the RNA/ethanol mixture. These were then mixed and frozen at -20°C overnight. The frozen mixture was spun at full speed (20,000 x g) in a standard microcentrifuge at 4°C for 30 minutes. The supernatant was decanted. The pellets were dried by air (tubes open) for 15 minutes. The pellets were then stored until shipment to Japan at temperatures above 20°C. At the time of analysis, the pellets were re-dissolved by addition of 50 µl of sterile RNAase-free double-distilled water per RNA sample, vortexed and spun down to re-suspend.

### **3.7 Procedures of RT-LAMP Analysis**

All the collected and extracted NDV RNA samples were analyzed by RT-LAMP (Notomi *et al.*, 2000; Mori *et al.*, 2001; Baba *et al.*, 2002). One set of the six optimized primers (four LAMP primers and two Loop primers) was used for the RNA amplification during RT-LAMP reaction. The RNA Amplification Kit (RT-LAMP), loopamp® (Eiken Chemical Co., Ltd., Bunkyo-ku, Tokyo, Japan) was used. The instructions of the manufacturer of the RT-LAMP Kit and Primers were strictly followed during use of the reagents.

The materials used during the reaction preparations were supplied in the kit and included 2 x Reaction Mixture [LAMP buffers containing Tris-HCl (pH 8.8): (40mM), KCl (20 mM), MgSO<sub>4</sub>: 16 mM, (NH<sub>4</sub>)<sub>2</sub>SO<sub>4</sub>: 20 mM, Tween 20: 0.2 %, Betaine 1.6 M and dNTPs: 2.8 mM each]; Enzyme Mixture containing two enzymes: AMV Reverse Transcriptase

and *Bacillus stearthermophilus* (Bst) DNA polymerase; Distilled water; Primer Mixture and Positive Control RNA and Other materials included: Dissolved RNA samples; Sterile tubes for master mixture preparation; Micropipettes; Pipette tips with filter; Loopamp reaction tubes; Aluminum rack for cooling tubes; Ice (crushed) and ice box; Microcentrifuge; Vortex mixer; Loopamp Real-time Turbidimeter (for real-time turbidity detection); and Loopamp Fluorescent Detection Reagent.

In order to conduct each reaction batch of reaction, a Primer mixture was first made. Using an optimized set of primers (Uga2) the Primer mixture was made by combining different specific primers at varying volumes: FIP at a volume of 40  $\mu$ l; BIP at 40  $\mu$ l; F3 at five microlitres and the volume of five microlitres for B3 primer of microlitres. The volume of the loop primers was 20  $\mu$ l for each of LF and LB primer. A fresh mixture was made each day a batch of RT-LAMP test reaction was to be conducted. After each mixture was made, it was stored at 4°C until analysis.

The second step involved making of a Master mixture. The mixture comprised the reagents combined; LAMP Buffers at a volume of 12.5  $\mu$ l, Primer Mixture at 1.3  $\mu$ l, Double Distilled Water (DDW) at 4.2  $\mu$ l, Test RNA at 5.0  $\mu$ l, Enzyme Mixture at 1.0  $\mu$ l and Fluorescent Dye (optional) at 1.0  $\mu$ l volume rate. A total of 25.0  $\mu$ l was the final volume of the reaction mixture.

In cases where the observations of the reaction results were to be made by methods other than UV light, the fluorescent dye was not added to the mixture. In such cases, the volume of DDW was increased by the equivalent volume (1.0  $\mu$ l) of fluorescent dye, not included in the mixture.

As a precautionary measure, whenever a Master mixture was being made, one additional reaction was considered to cater for any accidental/inevitable losses of solution during preparation of reaction mixtures. The total volume of the Master mixture that was made each time depended on the number of test reactions to be run. Consequently a suitable multiplication factor could be determined and used, and the final volume of Master

RT-LAMP amplification of the sample (RNA) was demonstrated by the turbidity-versus-time curve obtained using the real-time turbidimeter (Mori *et al.*, 2004).

### **3.8 Ethical considerations**

If cloacal and oro-pharyngeal cotton swabs (samples) were to be collected from living birds, this was done only once in a day. Otherwise, for all the experimentally infected chickens the tracheal tissues, cloacal and oro-pharyngeal swabs were collected from chicks shortly after death. In order to minimize trauma on chickens during slaughter, the humane method described by Hofstad (1984) was used. The collection of blood from the chicks was done using needles of gauge 22, only. Heart puncture was done with careful handling of the chicks at the time of procedural application and the chicks on which the procedure was applied were immediately sacrificed. The carcasses were disposed of packed in biosafety polythene bags and immediately incinerated.

### **3.9 Limitations**

Because of availability of limited funds for purchase of extraction reagents, only 240 samples of the 369 collected from the experimental chicks and only 156 samples of the 304 collected from the field Newcastle disease outbreaks were extracted and analyzed by RT-LAMP method. A total of 360 samples from the experimental chicks and 304 from the field chickens had initially been collected for analysis. Fortunately, the sample size for each part of study had been determined as 120.

## **Chapter 4**

### **Evaluation of Reverse Transcriptase Loop-mediated Isothermal Amplification for Detection of Newcastle Disease Virus in Non-tissue Samples in Chickens**

#### **Abstract**

A study to evaluate the use of non-tissue samples in Reverse Transcriptase Loop-mediated isothermal amplification (RT-LAMP) for detection of Newcastle disease virus was undertaken. This followed detection of the RNA in tissue samples in an earlier study.

Tracheal tissue and cloacal and oro-pharyngeal swab samples were collected from experimental chicks on station. Immunity against Newcastle disease (ND) in the chicks was monitored by Haemagglutination inhibition test. A total of 138 naïve chicks were inoculated with the velogenic strain of ND virus. Clinical signs among the chicks and postmortem lesions were recorded and infection confirmed by immunochromatographic assay prior to sample collection. Newcastle disease viral RNA extracted from 41 tracheal tissues, 92 cloacal swabs and 107 oro-pharyngeal swabs was analysed by RT-LAMP. Ten naïve chicks provided the control.

Clinical signs in infected birds were observed with morbidity rate of 100 % and mortality rate of 93.9 % by end of day seven post infection. RT-LAMP amplified RNA from 95.0 % of the tracheal tissues, 78.3 % of the cloacal and 41.1 % of the oro-pharyngeal samples. RT-LAMP preliminary analytical sensitivity was 0.85 pg for tracheal tissues, 3.5 pg for cloacal swabs and 25 pg for oro-pharyngeal swabs. The detection limit of 0.85 pg achieved in this study was slightly higher than the 0.5 pg reported in an earlier study in which tissue samples were used. Reaction time in 75.3 % of the samples (n=295) was from 20-36 minutes. Only 2 test reactions exceeded 45 minutes.

Since ND virus was detected from cloacal swabs with a fairly high rate by RT-LAMP, these swabs be adopted as a virus recovery specimen in the diagnosis of ND infection by this method. Further investigation, however, should be done on the use of oro-pharyngeal swabs in the detection of ND virus infection by this method.

*Key words: Newcastle disease virus, infection, chicks, RT-LAMP, analytical sensitivity and reaction time.*

#### 4.1 Introduction

Newcastle disease is the most important infectious disease affecting free-range village chickens, particularly in developing countries including Uganda (Aini, 1989; Mukiibi, 1992 and NARO, 1996). The disease is a highly contagious virus infection of poultry causing severe economic and production losses worldwide (Alexander 1988a). Newcastle disease is reportedly endemic in Eastern Uganda and assumed to be so in the rest of the country (Otim *et al*, 2004).

Laboratory diagnosis of ND is done through serological tests, establishment of biological properties of virus strains and molecular techniques (Calnek, 1997; Alexander, 1989; Alexander, 1997; and Kant *et al.*, 1997). The assays that have been used to identify NDV include Haemagglutination test and Haemagglutination inhibition (HI) test, virus neutralization, complement fixation, neuraminidase inhibition, haemolysis inhibition, and ELISA in domestic birds (Alexander, 1989; Jestin *et al.*, 1989; Wilson *et al.*, 1984). HI test is the most widely used test (OIE, 2004). In addition to HA test, other antigen-based diagnostic tests include Virus isolation (OIE, 2004), biological pathotyping (Alexander, 1997), PCR (Nested PCR and RT-PCR) (Jestin and Jestin, 1991; Jestin *et al*, 1992; Seal *et al*, 1995; Collins *et al.*, 1996; Marin *et al*, 1996; Kant *et al*, 1997; Lomniczi *et al*, 1998), RT-Nested PCR-ELISA (Kho *et al.*, 2000), Nucleic Acid Sequence Based Amplification (NASBA) and Sequence Displacement Amplification (SDA). Another recently developed test for rapid diagnosis of ND is chromatographic immunoassay (<http://www.anigen.co.kr>, 2005). Despite the availability of these tests, more convenient and rapid methods for the diagnosis of diseases are desired (IAEA/FAO, 2006).

Recently a novel nucleic acid amplification method termed Loop-mediated isothermal Amplification (LAMP) was developed (Notomi *et al.*, 2000). Results of a study by Pham and others (2005) specifically demonstrated that the LAMP-based assay is a useful tool in the rapid and sensitive diagnosis of NDV infection and that all LAMP-positive samples during the test were also positive by nested PCR. In their study, that involved, among others, the use of trachea and lung specimens, all the samples were positive by both RT-LAMP and nested PCR with detection limit as low as 0.5 pg during the comparison of the

analytical sensitivity of the two tests. Both tests were positive in samples with the target gene and negative in all the samples with non-target genes in 100 % of the samples tested, exhibiting a high and equal level of specificity by the two methods (Pham *et al.*, 2005).

Since RT-LAMP had demonstrated its ability to detect ND viral RNA from tissue samples with a high analytical sensitivity and specificity (Pham *et al.*, 2005), the use of less invasive (non-tissue) samples should be evaluated. The use of tissue samples, which involves sacrificing a chicken for diagnosis for use in developing countries, is commonly resented by farmers.

There are a number of ways to evaluate a diagnostic test, varying from technical studies of calibration to controlled trials of the effect upon patient outcome (Martin *et al.*, 1987). However, evaluation of a diagnostic test usually refers to measurement of the diagnostic parameters of the test: sensitivity, specificity, positive and negative predictive values, and likelihood ratios (Martin *et al.*, 1987). In the immunologic, pharmacologic and analytical usage, sensitivity of a test refers to the ability of the test to detect a small amount of, among others, the antibody and antigen (Martin *et al.*, 1987). In analytical sensitivity, the more sensitive the test, the smaller the amount of antigen (for example) it will detect (Thrusfield, 2001). Analytical sensitivity as a parameter of validity of a diagnostic test is termed diagnostic sensitivity (Stites and Rodgers, 1991). The sensitivity and specificity of a diagnostic test are important in deciding the value of the test in disease control campaigns and in categorizing animals in observational studies (Thrusfield, 2001).

## **4.2 Materials and methods**

### **4.2.1 Sources of experimental materials**

This study was conducted using experimental chicks on station and sick chickens under free-range system from an area with a reported outbreak of Newcastle disease. In the on-station experiment chicks were kept from day-one upto the 5<sup>th</sup> week, at which age, they had no or very low maternal antibody titres against Newcastle disease, by HI test ( $\leq 1$  Log2). Chickens for sample collection in the field were selected from Newcastle disease *Anigen Rapid NDV Ag Test*<sup>®</sup> (chromatography) test-positive identified flocks. The chicks

for on-station experiment were male hyssex chicks obtained from Bukomo Breeders (Uganda) Company. They were obtained at the age of day-one and kept in an on-station experiment at the National Livestock Resources Research Institute (NaLIRRI), Tororo.

#### **4.2.2 Viral strains used for inoculation**

Allantoic fluid containing 16 velogenic Newcastle disease virus isolates from Eastern Uganda (Otim, 2004) and non-characterized field-collected velogenic strain Newcastle disease virus obtained from samples collected from areas of Newcastle disease outbreak were used for inoculation. Chromatography and HI test confirmed-positive samples were the sources of the field collected NDV. The samples collected from the field were also processed (virus isolation in 4.2.3) before being used for egg inoculation. The two categories of NDV source were independently passaged, twice, in local embryonated eggs obtained from non-vaccinated chickens. Embryonated eggs were used because these are a convenient source of rapidly growing cells within which most avian viruses will readily propagate (OIE, 2000). Local embryonated eggs were used as opposed to specific pathogen-free (SPF) eggs, as they were the only type available. Passaging was done twice in eggs to increase infectivity of the viruses.

#### **4.2.3 Virus isolation**

The processing of the Newcastle disease positive field-collected samples involved slicing tracheas and mincing of others in separate sterile mortars using sand and pestles, addition of 3 – 5 ml of phosphate buffered solution and addition of antibiotics, namely: gentamycin, penicillin and streptomycin at the rate of 50 µg per ml, 2,000 units per ml and 2 mg per ml, respectively, at pH 7.0 – 7.4 (OIE, 1996). This was accompanied with centrifugation at 1,000 rpm for 10 minutes and 0.2 ml of the supernatant (fluid) used as inoculum for the eggs.

The embryonated eggs used for inoculation were incubated in a conventional egg incubator at a temperature of 37.4°C at a relative humidity of 60-65 % and with egg turning facility. Before incubation the eggs were incubated for nine days and candled for the purpose of identifying those that were fertile. The candling was done on a daily basis

from day four of incubation. The infertile eggs and those with floating air cell or, in which the embryos appeared weak, were discarded.

At the time of inoculation, the egg shells were disinfected at the site of inoculation using a solution of 70 % (v/v) ethanol before and after inoculation for the purpose of avoiding/minimizing contamination. A hole was drilled in the shell of each of the eggs a small dental drill. The inoculation into the egg was done at the reference point for inoculation, marked with a pencil, 2 – 3 mm on the side of the air sac, away from the border with the air space and the chorioallantoic membrane (CAM). A sterile disposable 1-ml syringe fitted with a 23-gauge, sterile 1-inch long needle was used for inoculation of each egg. During inoculation the needle was pushed perpendicularly through a hole already drilled, about 3 cm to avoid injuring the growing embryo in the egg. Inoculation was done through the CAM. Immediately after inoculation each egg was covered with a cellotape to prevent possible contamination while in the incubator. The inoculated eggs were immediately returned into the incubator. Sterile scissors and scalpel blades were used during the procedure.

The incubating eggs were monitored for any embryonic activity and mortality on a daily basis post inoculation by candling them in a dark room until after death of embryo or termination of incubation after the 5<sup>th</sup> day of inoculation. Any loss of activity, mortality and/or loss of viable blood vessels during the 1<sup>st</sup> day post inoculation was regarded to have been caused by trauma or contamination. Embryonic death 3 – 5 days post inoculation was attributed to presence of viable Newcastle disease virus. Death, three days post infection could be an indicator of presence of a virulent virus (OIE, 2004).

Harvesting of the allantoic fluid was done following death of the embryo in the virus-inoculated egg. The egg intended for harvesting was first removed from the incubator and kept at about 4°C overnight to avoid bleeding (seen as red blood cells in the allantoic fluid) during harvesting (OIE, 2004). On harvesting the allantoic fluid was first subjected to slide agglutination test and chromatographic immunoassay in order to confirm the

presence of NDV. The test-positive allantoic fluid was immediately aliquoted and kept at  $-20^{\circ}\text{C}$  until time of inoculation or other future use.

#### **4.2.4 On-station experimental design**

Chicks were introduced at day one of age. They were kept in one group for upto five weeks of age, the period, during which, they were monitored for their immune status against ND. The chicks were not vaccinated against any disease during the period except treating them against coccidiosis using anti-protozoan drugs. Multivitamins were provided continuously-almost throughout their stay. At the age of five weeks when the chicks were tested to have no or extremely low ( $<1 \text{ Log}_2$ ) HI test titres, they were inoculated with NDV allantoic fluid.

As an initial step of evaluating the infectivity of the virus in the allantoic fluid that was intended for infecting the chicks, 5 different naïve chickens at the age 8 weeks were inoculated with this fluid. All chickens began manifesting ND signs by the 5<sup>th</sup> day of infection. They all died on the 7<sup>th</sup> day with severe ND clinical status. The lesions seen at postmortem and the antigen-based tests (chromatographic immunoassay on cloacal swabs) confirmed the cause of death as ND.

#### **4.2.5 Sample collection**

Various types of samples were collected during the study. Tracheal tissues, lungs and spleens were, first collected from the field for ND virus isolation. Blood (for serum) was collected for HI test, cloacal swabs were collected for chromatographic immunoassay (*Anigen test*) during confirmation of ND outbreak reports and tracheas, cloacal and oropharyngeal swabs for RT-LAMP analysis.

Samples for RT-LAMP assay were collected, at all times, at peak of manifestation of clinical signs or immediately after death from the Newcastle disease. This was done in both cases – be it on-station or during field sample collection.

The method of blood collection included use of heart puncture (for chicks from the age of day one to three weeks) and bleeding using the wing web vein for birds above three weeks of age. Bleeding by heart puncture was done humanely including use of a large gauge (22) needle and collecting as much blood as possible. The bled chicks were then sacrificed immediately.

Ninety five (n=240) of samples on-station were collected from birds immediately after death. However, for samples collected from the field, samples for virus isolation were obtained; the slaughter, postmortem, examination and sample collection procedures described by Hofstad (1984) and Fowler (1996) were used. A thorough postmortem was done on 45.6 % (n=138) of the infected chicks. Postmortem was done purposely to observe and record gross lesions in the sampled cases as a test to confirm that pathological lesions seen were similar to those reported of NDV infection (Calnek *et al.*, 1997).

In addition to the clinical signs in live chickens and gross lesions observed at postmortem, infection was also confirmed after cloacal samples from some for the chickens in a given flock tested positive to *Anigen Rapid NDV Ag Test*<sup>®</sup>.

#### **4.2.6 Procedures used in Haemagglutination Inhibition Test (OIE, 2000)**

The test involved two steps. The first step was Haemagglutination (HA) test and the second, HI test. The HA test was aimed at testing to confirm the ND virus and to determine the titre for diluting the antigen during the time of preparing for the HI test reactions. The presence of the antigen was exhibited by causing haemagglutination in the HA test reaction mixture, seen on the sides at the bottom of the microtitre plate well(s).

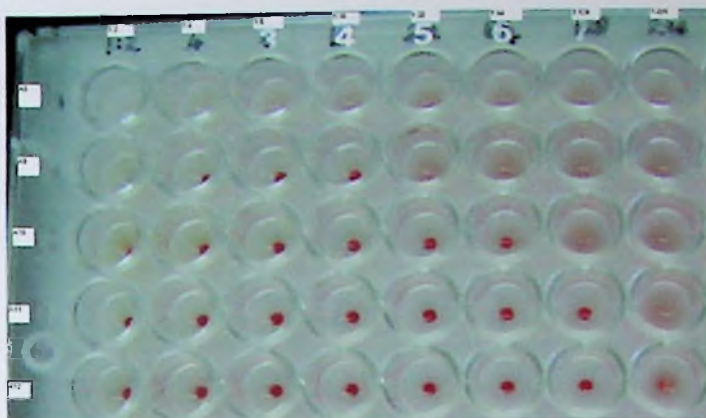
The antigen suspension used in this study was diluted by two-fold transfers. A back-dilute of the antigen suspension with the cell suspension used in the HI test was done to be certain that the HI activity was actually as calculated (Allan and Gough, 1974).

In HI test, the antigen was added to the serum and the antigen-serum mixture dilutions are made. In this antigen-added method, the antigen diluted so that desired number of HA units in the final volume of the diluted serum together with the added antigen was reached before the red blood cells were added (Beard, 1984).

Blood samples for purpose of establishing the immune status of the chicks were analyzed by HI test. Analysis by HI test involved the use of U-bottomed microtitre plates, micro pipettes, Phosphate Buffered Saline (PBS), Newcastle disease virus (confirmed antigen), the test serum and serum obtained from chickens free from Newcastle disease (for negative control) and positive Newcastle disease antiserum (for positive control) (Veterinary Laboratories Agency, New Haw, Addlestone, Surrey KT15 3NB, UK).

The chicken red blood cells used for HA and HI tests were got by bleeding chickens that had never received infection or been vaccinated against Newcastle disease virus for over 6 years. The blood was collected in Acid Citrate Dextrose (ACD) solution (prepared in the laboratory at NaLIRRI) at a ratio of 1:3 of the solution to blood, respectively. Chicken blood cells were washed using Dextrose Gelatin Veronal (DGV) solution (prepared in the laboratory at NaLIRRI) at a ratio of blood to DGV of 1:9, respectively. The cells were washed three times with centrifugation at a speed of 1,500 rpm for 10 minutes using Centaur 2 MSE ® (Fisons, England) centrifuge. After their preparation, washed chicken red blood cells (10 % cells) were kept at 4°C until use; and were used within 7 days from the day of blood collection. At the time of use, a 1 % red blood cell mixture was prepared using PBS for immediate use in HA or HI test reaction mixture. The preparation procedures for ACD and DGV solutions are as indicated in Appendix 1.

Haemagglutination inhibition test was conducted, on each batch of collected sera for the purpose of establishing the presence of antibodies against Newcastle disease in the test sera. Inhibition of haemagglutination in the test reaction mixture indicated the presence of antibodies against ND. This was observed as red blood cells setting to form a button at the bottom of the well(s) (Figure 4.1).



**Figure 4.1. Haemagglutination Inhibition test plate** (Source: Otim *et al.*, 2004)

Quantities of the various reagents and test materials, the serial dilutions, timing and precautions as described by Allan and Gough (1974) in the procedure of ND diagnosis by HI were followed.

#### **4.2.7 Inoculation of chicks with virus**

On the 3<sup>rd</sup> day in the 5<sup>th</sup> week, when the HI titres (immunity) had almost completely dropped to 0Log<sub>2</sub>, test chicks were inoculated intranasally and by the ocular route with the velogenic strain of NDV obtained from Eastern and Central Uganda. About 0.3 ml of a 10<sup>-1</sup> concentration of inoculum was used. In a study involving inoculation of experimental chicks with NDV, Squibb (1964) infected chicks at the age of 28 days (four weeks), when the immunity was observed to have dropped. The dose of inoculum used at study was 0.1 ml of a 10<sup>-1</sup> concentration of NDV, as the targeted level of mortality was only 50 %. The chicks were then monitored for manifestation of clinical signs.

#### **4.2.8 Sample processing and preservation**

Blood samples were left on the bench overnight and sera separated the following day. The serum was either used for HA or HI tests immediately or preserved under refrigeration at - 20°C until use. Other samples were processed as described in Chapter 3.

#### **4.2.9 Sample size determination**

The sample size was determined based on Bachmann and others (2006) in whose study it was revealed that the median sample size for studies on diagnostic accuracy was 117.

Also considered was McNemar's test requires at least 20 matched samples to be used. A total of 240 samples were collected from experimental chicks for the purpose evaluating the RT-LAMP diagnostic technique on non-tissue samples. These included 41 tracheal tissues, 92 cloacal swabs, 107 oro-pharyngeal swabs and eight negative control samples (tracheal tissues). The samples were from 107 chicks.

#### **4.2.10 Sampling method used**

Before inoculation with the Newcastle disease virus, on-station experimental chicks were regularly tested by HI test for presence of antibodies against Newcastle disease. This was for the purpose of establishing the baseline on the immune status of the chicks against Newcastle disease. Ten per cent of the chicks at the age of one week, two weeks and at the end of the 3<sup>rd</sup> and 4<sup>th</sup> week were bled. A total of 82 blood samples were collected. Chicks for blood collection were randomly picked from the rest of the flock. This was done, first by picking one chick from the flock and then thereafter, every 10<sup>th</sup> chick to emerge from the cage was picked. This continued until 10 % of the chicks in the unit were finally picked. Chicks were continuously made to spread in the cage, as they were forced out, during the entire selection procedure, each time bleeding was done.

For the samples intended for RT-LAMP assay, cloacal and oro-pharyngeal swabs were collected only from experimental chicks that had died while in the clinical state of Newcastle disease following inoculation with NDV. Tracheal tissue samples were collected after postmortem examination. Only carcasses with ND postmortem lesions such as haemorrhages in the trachea and proventriculus and petechial haemorrhages in the heart muscles were considered for trachea collection. The samples were immediately labeled based on the study name (L for LAMP), tag number and organ from which each was collected.

#### **4.2.11 Procedures used during RNA extraction from samples**

Ribonucleic Acid (RNA) from the tracheal tissues and cloacal and oro-pharyngeal swabs was extracted by use of the QUAamp® Viral RNA Mini Kit (Quagen, 2005). The procedure (materials, reagents and procedures) as described in Chapter 3 were followed.

4.2.12 Designing and optimizing of Primers

Oligonucleotide primers used for LAMP assay were designed from the target gene (fusion [F] gene) using one of the 16 Newcastle disease virus isolates from Eastern Uganda (Otim, 2004) based on the regions of similarity. The primers were using the Primer Design Explorer (4) of Eiken Chemical Co. Ltd (2007).

Table 4.1. Primer sets designed/used for the study

| No                                     | Oligo Name | Len | Tm   | GCrate | Sequence (5' – 3')                               |
|----------------------------------------|------------|-----|------|--------|--------------------------------------------------|
| <b>A. Designed primer set 1 (Uga1)</b> |            |     |      |        |                                                  |
| 1.                                     | F3-Uga1    | 20  | 72.5 | 0.65   | ACACTCATGGGGGCCGAAGG                             |
| 2.                                     | B3-Uga1    | 21  | 69.1 | 0.57   | CTTGCTGATGCCTGACAAGGG                            |
| 3.                                     | FIP-Uga1   | 40  | 68.9 | 0.53   | GATACAATAAGGCGGGGGAGGAGTTCTCACAGTAGGGACA         |
| 4.                                     | BIP-Uga1   | 38  | 68.3 | 0.53   | GACAGTCTATAACAAAACCTGCCTGGCCGGGTGAAAGC           |
| 5.                                     | LF-Uga1    | 21  | 62.4 | 0.52   | GAAGACCCTCGTTGGTACAAG                            |
| 6.                                     | LB-Uga1    | 23  | 52.4 | 0.39   | GCTACTCTTCATAGTCCTTATAC                          |
| <b>B. Primer set 2 (Uga2)</b>          |            |     |      |        |                                                  |
| 1.                                     | F3-Uga2    | 16  | 65.7 | 0.69   | CAAGCCTGGGCGGTTC                                 |
| 2.                                     | B3-Uga2    | 19  | 65.4 | 0.53   | ACAATAAGGCGGGGGAGAA                              |
| 3.                                     | FIP-Uga2   | 40  | 71.0 | 0.58   | CGGGTCCTCACCCAAAGATGTTGGTGGAAAGCGCGTACAG         |
| 4.                                     | BIP-Uga2   | 40  | 71.0 | 0.58   | ACACTCATGGGGGCCGAAGGTGAAGACCCTCGTTGGTACA         |
| 5.                                     | LF-Uga2    | 22  | 53.9 | 0.32   | ACACTTTGATGGATAAGATAAC                           |
| 6.                                     | LB-Uga2    | 20  | 56.9 | 0.50   | CAGAGTTCTCACAGTAGGGA                             |
| <b>C. Primer set 3 (Uga3)</b>          |            |     |      |        |                                                  |
| 1.                                     | F3-Uga3    | 18  | 60.8 | 0.50   | GCCCTTGAATCTTCGTTG                               |
| 2.                                     | B3-Uga3    | 20  | 55.7 | 0.40   | AGGGTAGAATGATGTGACAT                             |
| 3.                                     | FIP-Uga3   | 44  | 67.2 | 0.45   | CGCAGCCCTGTTAATTTGATAAGAGGCGCTGCTAAATACTGAGT     |
| 4.                                     | BIP-Uga3   | 47  | 75.1 | 0.40   | GCCTGTTTCATGACCCAGATTATATACTAGTGTCGTCTACTATGAGTT |
| 5.                                     | LF-Uga3    | 17  | 50.6 | 0.41   | GAGACGTTATTGCATTG                                |
| 6.                                     | LB-Uga3    | 15  | 63.2 | 0.67   | CGGGGGGATAGGCAA                                  |

- Oligo Name –Oligonucleotide primer name;  
Tm - Melting temperature of the Oligonucleotide;  
Len - Length of the Oligonucleotide; and  
GCrate- Percentage of the base CG base pairs along the Oligonucleotide

A total of three sets of primers each consisting of four LAMP Primers and two Loop Primers were designed and ordered from SIGMA (2007). For the purpose of selecting the best primers of the three sets (Table 4.1), all the three primers sets were tested for

sensitivity. One tracheal tissue (RNA) sample, known to be positive (positive by HA test and Chromatographic immunoassay), was used for determination of the sensitivity of these primer sets. A primer mixture for each set of primers was made. Using the positive tracheal sample, three reaction mixtures, each containing a different set of primer, were made. The LAMP tubes with the various reaction mixtures were then placed in different wells in the different blocks of the real-time Turbidimeter. The wells were set to run at different reaction temperatures. Table 4.1 shows the different primer sets designed in this study and Appendix 2 shows the results of RNA amplification by the different primer sets.

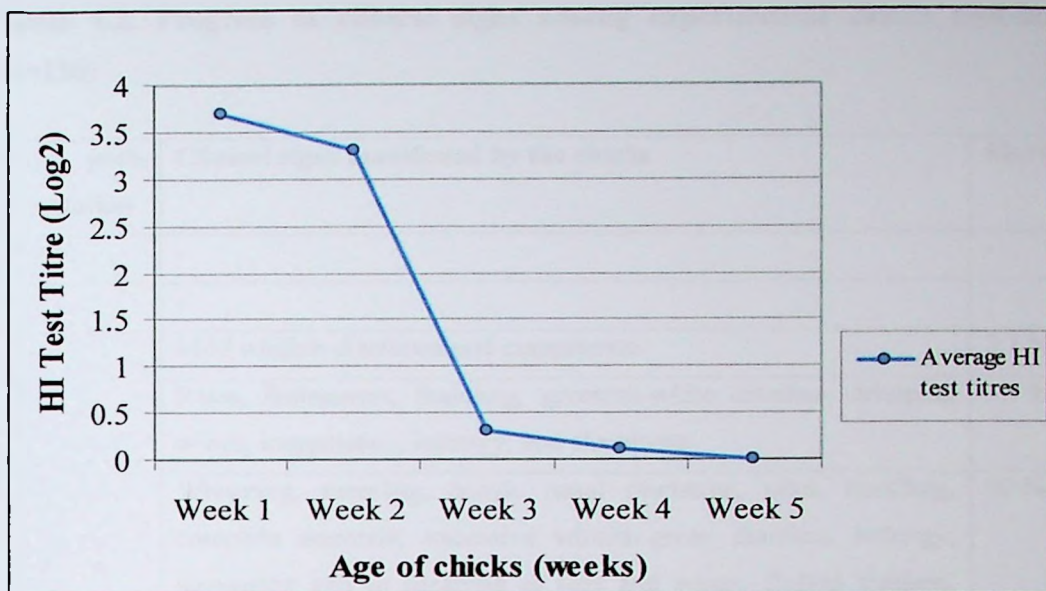
The temperatures 64°C and 65°C, were selected for further optimization of the primers of reaction temperature and time. The reaction time was 90 minutes.

#### **4.2.13 Procedures of RT-LAMP Analysis (Notomi *et al.*, 2000; Mori *et al.*, 2001; Baba *et al.*, 2002).**

The NDV RNA extracted from the 240 samples was analyzed by RT-LAMP. The set of the 6 optimized primers (four LAMP primers and two Loop primers) selected for further optimization was used for the RNA amplification. The RNA Amplification Kit and the LAMP test procedures described in the Chapter 3 were used in this study.

#### **4.2.14 Agarose gel electrophoresis for detection of LAMP products**

For the purpose of determining sensitivity of primers, primer optimization agarose gel electrophoresis was done. Agarose gel was prepared, placed in the electrophoresis tank (Horizontal) filled with x1 TAE (Tris Acetic acid EDTA) buffer. The gel was then loaded with the marker (100 kbp), the sample reaction mixtures (after incubation) and negative and positive control. The horizontal tank was then connected to a power (100 V) source and switched on. The system was electrophoresed for 30 minutes and powered off. The gel was then incubated with a staining solution (Ethidium bromide) for 15 minutes and multiple bands were visualized by picture taken in presence of UV light through the Gel documentation system, including taking and printing the picture.



**Figure 4.2. Trend of Haemagglutination Inhibition test titres against Newcastle disease among on-station experimental chicks**

#### **4.3.2 Clinical signs manifested by experimental chicks**

Following inoculation with the velogenic strain of NDV the chicks did not manifest any clinical signs of Newcastle disease during the first two days post-inoculation; they all appeared apparently healthy. Table 4.2 presents the clinical signs observed among chicks post-inoculation. Mild signs were seen at the start of the clinical picture. The signs became more severe during day 5 – 7 of the infection. Morbidity and mortality rates of 100 % and 68 % had been recorded by the end of the sixth day. By day 7, the time at which all samples required had been collected and hence discontinued sample collection, the cumulative mortality rate among the chicks had reached 87.2 %.

A cumulative mortality rate of 94 % was realized by the end of day 8 post-inoculation. Only nine chicks survived in the entire flock of experimental chicks. No clinical sign was observed among the 10 similar chicks that were used as control.

**Table 4.2. Progress of clinical signs among experimental chicks post-infection (n=138)**

| Day post-inoculation | Clinical signs manifested by the chicks                                                                                                                                                                                                                                                                          | Morbidity |
|----------------------|------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------|
| Day 1                | -                                                                                                                                                                                                                                                                                                                | -         |
| Day 2                | -                                                                                                                                                                                                                                                                                                                | -         |
| Day 3                | Mild whitish-diarrhoea and inappatence                                                                                                                                                                                                                                                                           | 2.1 %     |
| Day 4                | Rales, listlessness, huddling, greenish-white diarrhea, drooping wings, inappatence, lethargy, and sleepiness                                                                                                                                                                                                    | 52 %      |
| Day 5                | Wheezing, sneezing, cough, nasal discharge, rales, huddling, complete anorexia, excessive whitish-green diarrhea, lethargy, staggering gait to paralysis of legs and wings, fluffed feathers, weight loss, , torticollis and mortality of 20 %.                                                                  | 90 %      |
| Day 6                | More severe wheezing, sneezing, cough, nasal discharge, rales/difficult respiration, huddling, complete anorexia, excessive greenish diarrhea, lethargy, paralysis of legs and wings, fluffy feathers, weight loss, torticollis among surviving chicks and cumulative mortality of 68 %.                         | 100 %     |
| Day 7                | More severe wheezing, sneezing, cough, nasal discharge, rales/difficult respiration, huddling, complete anorexia, excessive whitish-green diarrhea, lethargy, paralysis of legs and wings, fluffy feathers, weight loss, staggering gait, torticollis among surviving chicks and cumulative mortality of 87.2 %. | 100 %     |
| Day 8                | Less severe signs of wheezing, sneezing, cough, nasal discharge, rales, huddling, inappetence, h-green diarrhea, lethargy, paralysis of legs and wings, fluffy feathers, weight loss, staggering gait, torticollis among surviving chicks and cumulative mortality of 94 %.                                      | 100 %     |

#### **4.3.3 Lesions observed at postmortem following death of the infected chicks**

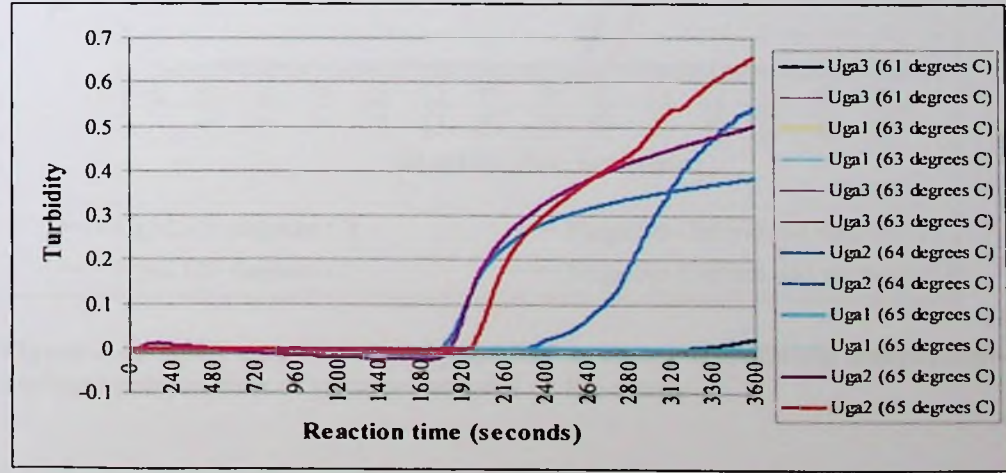
Lesions observed during postmortem included haemorrhages and necrosis in the gastrointestinal tract, especially on the peritoneal adipose tissue and serosal lining, haemorrhages in the tracheal rings of cartilage and the mucosa of the proventriculus.

Other lesions were petechial haemorrhages on the serosa of an enlarged spleen and petechial haemorrhages on the tip of the heart muscle. All these lesions are typical to those indicated in Newcastle disease infection (Geering and Forman, 1995; Calnek *et al.*, 1997). Of the 63 cases where postmortem was done, 90.5 % of them had all the lesions reported in this study. The rest only had petechial haemorrhages in the proventriculus and petechial haemorrhages on the serosa of an enlarged spleen.

**4.3.4 Optimizing of primers used in the study**

When the three designed primer sets were tested at the temperatures of 61°C, 63°C, 64°C and 65°C, in different wells of a real-time turbidimeter, Uga1 and Uga3 did not amplify ND viral RNA. Only Uga2 amplified the RNA. The amplification reaction of the ND viral RNA from the tracheal tissue sample was achieved at the temperature of 64°C and 65°C.

The primer set, Uga2, was selected as a better set since it amplified the viral RNA at temperature of 64°C in 32:30 minutes (well C1) and 45:40 minutes (well C2); and at 65°C in 32:30 minutes (well D3) and 34:41 minutes (well D4). Figure 4.3 and Appendix 2 show the amplification results of the different primer sets at different reaction temperatures. Of the three primer sets subjected to RT-LAMP reaction at the different temperatures, Uga2 was selected for further optimization on the suitability of reaction temperature.



**Figure 4.3. Reaction of different primer sets at different temperatures to a similar positive tracheal tissue RNA sample**

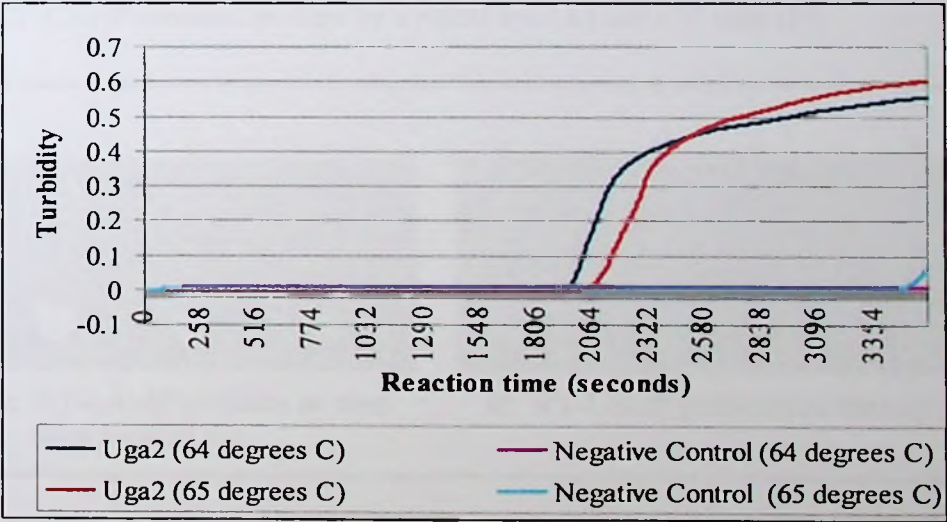
In a test conducted for further optimization of the primer set, Uga2, the amplification results are as shown in Table 4.3. In this optimization test, the primers set amplified the ND virus positive sample (tracheal tissue RNA).

**Table 4.3. RT-LAMP reaction on Newcastle disease viral RNA using Uga2 Primers**

| Heat Block | Well No. | Sample           | Reaction Temperature | Results   |
|------------|----------|------------------|----------------------|-----------|
| A          | 1        | Positive sample  | 64°C                 | 33:48(+)  |
|            | 2        | Negative control | 64°C                 | 0:00 (-)  |
| B          | 1        | Positive sample  | 65°C                 | 36:00 (+) |
|            | 2        | Negative control | 65°C                 | 0:00 (-)  |

+ = Positive and - = Negative

Results in Figure 4.4 and Table 4.3 indicate that Uga2 amplified the RNA in the sample at a temperature of 64°C and 65°C. The amplification was, however, better at the temperature of 64°C at which the reaction time was shorter (33:48 minutes) as compared to 65°C, at which time reaction occurred but slightly later (36:00 minutes).



**Figure 4.4. RNA amplification by LAMP primers at different temperatures during optimization of primer set used in the study**

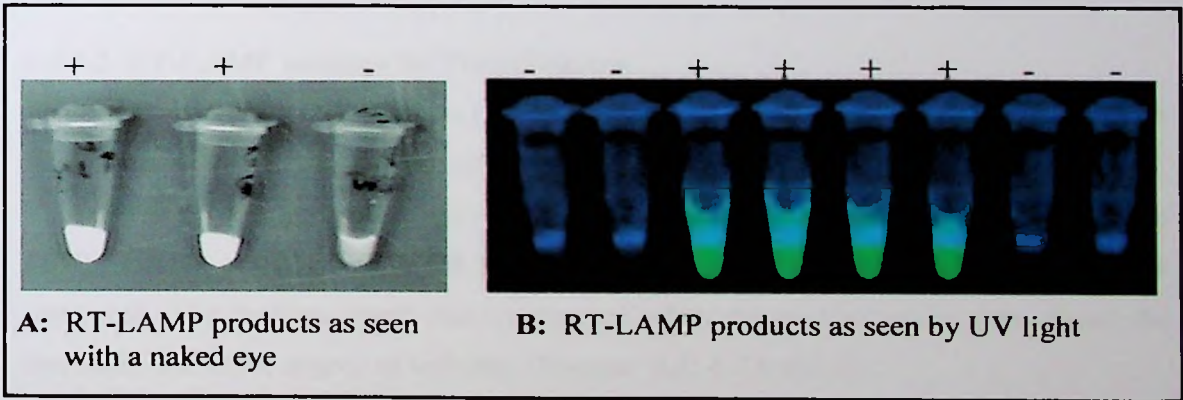
At both reaction temperatures this primer set did not amplify the negative controls. Based on the reaction results of this optimization test, the temperature of 64°C was finally adopted as the most suitable RT-LAMP reaction temperature for use in the study.

**4.3.5 Use of RT-LAMP in the detection of Newcastle disease viral RNA**

After selection and optimization of a suitable LAMP primer set, this primer set was used for the detection of RNA extracted from trachea (tissue) and cloacal and oro-pharyngeal swabs (non-tissue) samples. Some of the samples were only incubated in a heat block and observation made with naked eye or by use of UV light. The reactions of other samples were made in a real-time turbidimeter. In all cases of the different apparatus used for RT-LAMP in the study amplification products were observed and deductions made.

**4.3.5.1 RT-LAMP reaction by use of heat block**

Test reactions involving a total of 101 samples (Table 4.4) were prepared and incubated in a thermocycler (GeneAmp, PCR System 9700, Applied Biosystem, USA), as a heat block. In this machine samples were incubated for 180 minutes. The results of these test samples were observed by use of the naked eye. Figure 4.5 shows the appearance of the RT-LAMP products as seen by a naked eye (A) and UV light (B).



**Figure 4.5. Amplified RT-LAMP products as seen with a naked eye (A) and UV light (B)**

Test reaction mixtures were incubated and products observed within three minutes after being taken out of the PCR machine at the end of reaction. The presence or absence of turbidity was the basis for deducing that a sample in the reaction mixture was positive or

negative, respectively, whenever the naked eye was used. A turbid RT-LAMP product was regarded positive and a non-turbid (transparent) one, negative (Notomi *et al.*, 2000). In cases where UV light was used, the presence of green-coloured product indicated a positive result (Baba *et al.*, 2002) and a blackish product was interpreted as negative. The observation and result interpretation were generally easy by the two methods.

Summary results of all the reactions obtained by these methods of incubation and observation were as shown in Table 4.4 (Appendices 3, 4 and 5 provide the details of the test reactions). From the two reactions batches incubated by this method, 100 % (n=17) of the trachea samples tested positive, 100 % (n=16) cloacal samples and only 7.9 % (n=58) of the oro-pharyngeal samples tested positive.

**Table 4.4. Results of RT-LAMP on RNA incubated in a heat block and observed by a naked eye or by use of UV light**

|    | Sample types         | Positive results | Negative results | %age of positive results |
|----|----------------------|------------------|------------------|--------------------------|
| 1. | Trachea              | 17               | 0                | 100.0                    |
| 2. | Cloacal swabs        | 0                | 16               | 0.0                      |
| 3. | Oro-pharyngeal swabs | 05               | 58               | 7.9                      |
| 4  | Negative control     | 0                | 2                | 0.0                      |

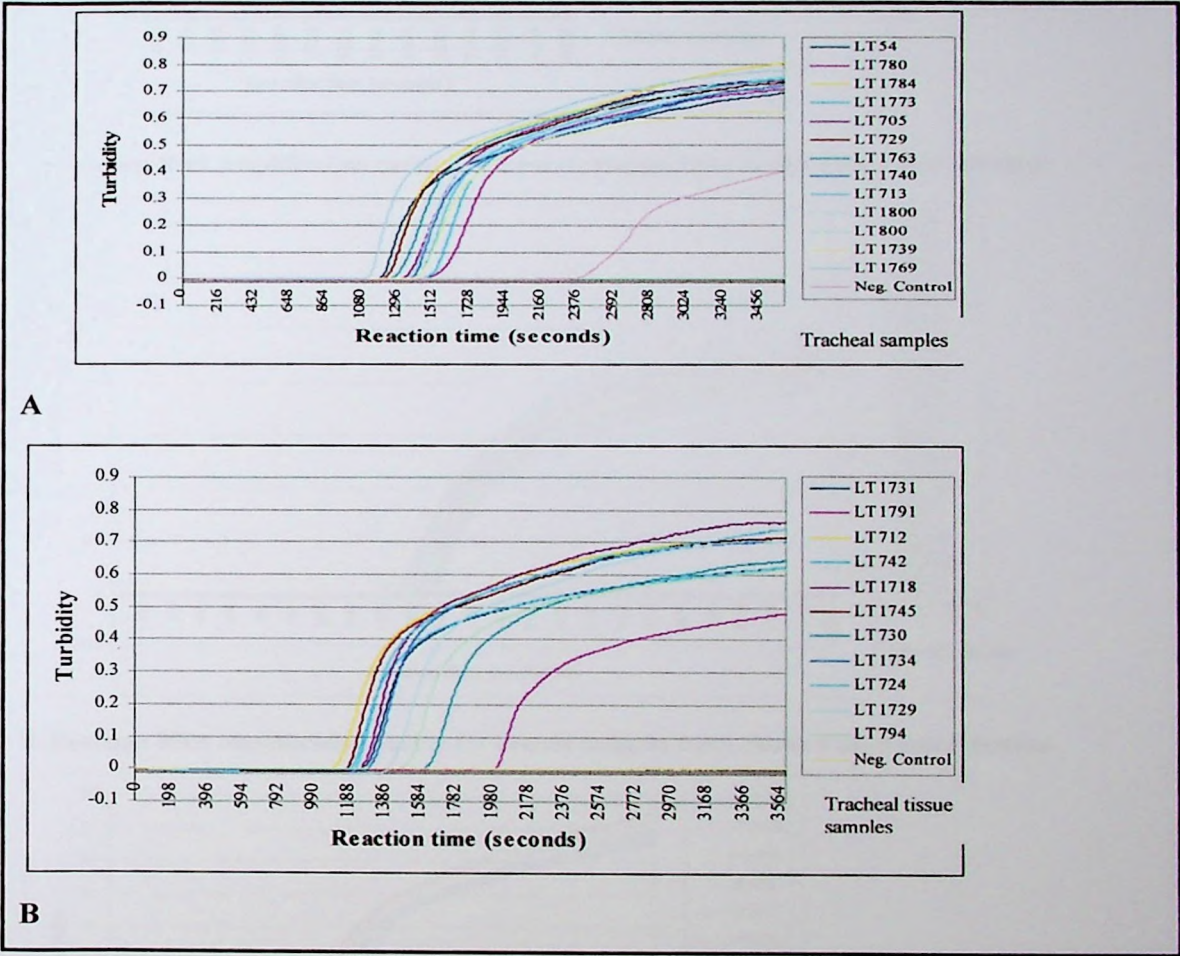
#### 4.3.5.2 RT-LAMP reaction by Turbidimetry

The test reactions of most samples (144 out of 240) were by real-time turbidimetry. With this method the time of RNA amplification for positive reactions was easily observed. The analysis result for each sample per batch (of samples) was plotted as melting curve for RT-LAMP reaction. A graph presenting the curves of all samples in a batch was generated. The positive result was interpreted when the melting curve rose above the threshold line at 0.1 degree of turbidity (Figures: 4.6; 4.7 and 4.8).

##### 4.3.5.2.1 RT-LAMP reaction using tracheal tissue samples

Ribonucleic acid (RNA) extracted from 24 tracheal samples collected from the chicks were analyzed by RT-LAMP method. The analysis was conducted in two batches. Figure 4.6 shows the results (of the two batches) of RNA amplification by use of the loopamp

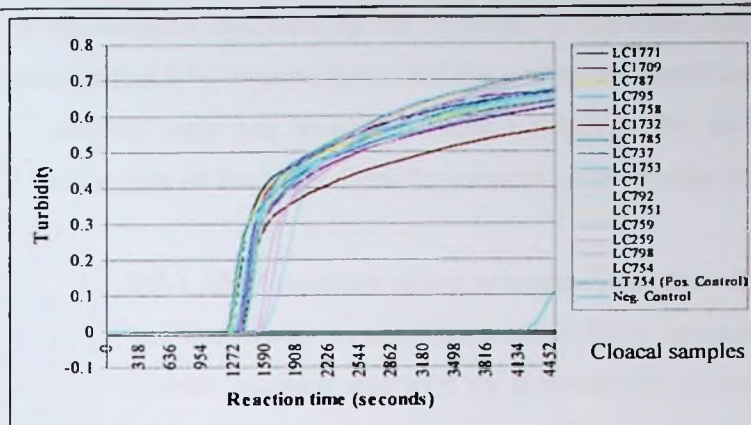
real-time turbidimeter. Of the 24 tracheal samples amplified in the both batches by this method, only RNA of one sample (LT 800) was not amplified. The results of the reaction provided a clear indication of the high rate of detection of RT-LAMP method on tracheal tissue samples. This high detection rate of Newcastle disease virus RNA by the method was also reported by Pham and others (2005), in a study in which the sensitivity and specificity of LAMP were compared to those of nested PCR.



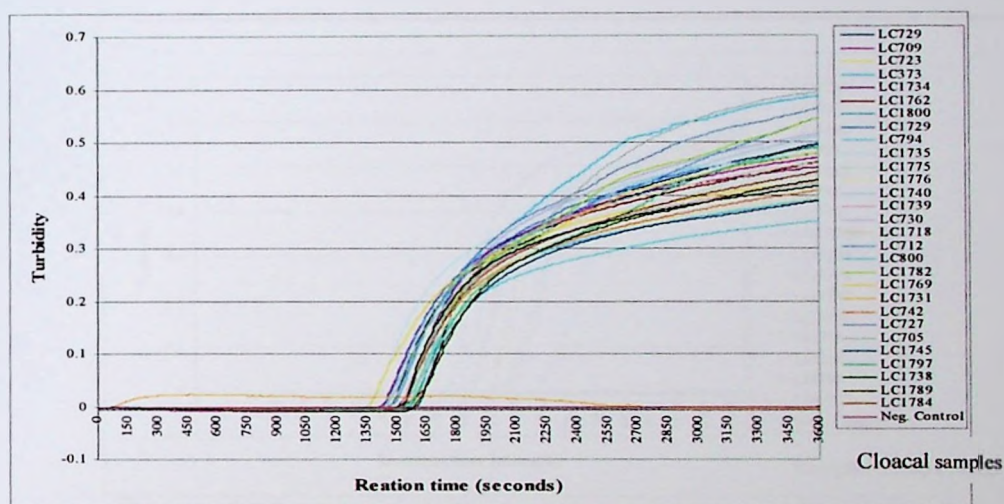
**Figure 4.6. RT-LAMP reaction (A and B batches) during amplification of RNA extracted from tracheal samples**

#### 4.3.5.2.2 RT-LAMP reaction using cloacal swabs

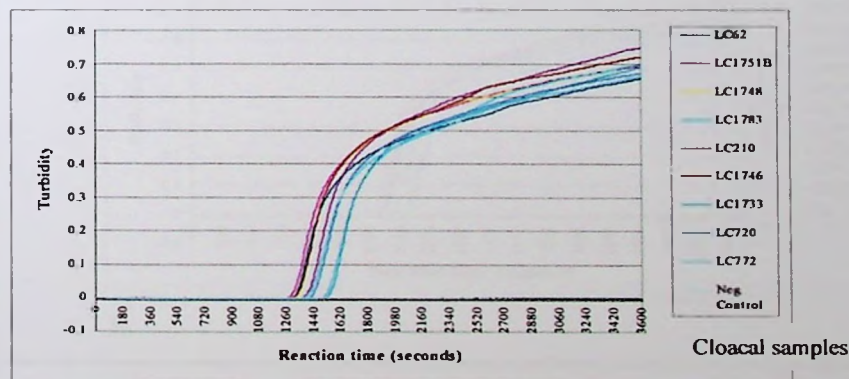
Seventy six of the cloacal swabs (samples) collected from the chicks were analyzed by real-time turbidimetry.



**A. Reaction RNA amplification curves for cloacal samples from chicks 6 days post infection**



**B. Reaction RNA amplification curves for cloacal samples from chicks 5 days post infection**



**C. Reaction RNA amplification curves for cloacal samples from chicks 6 days post infection**

**Figure 4.7. RT-LAMP reaction (A, B and C batches) during amplification of RNA extracted from cloacal swabs**

Of the samples analyzed by the method, in the three batches in the figure, RNA of 72 samples (94.7 %) was positive. All the negative control samples used in the three batches of analysis were not amplified (tested negative) by this method. This shows a high detection rate of the NDV from the cloacal swabs by the method of RT-LAMP.

4.3.5.2.3 RT-LAMP reaction using oro-pharyngeal swabs

In Figure 4.8 the results of amplification of RNA extracted from 27 out of the 44 oro-pharyngeal swab samples analysed by turbidimetry (some which results have not been shown) are presented.

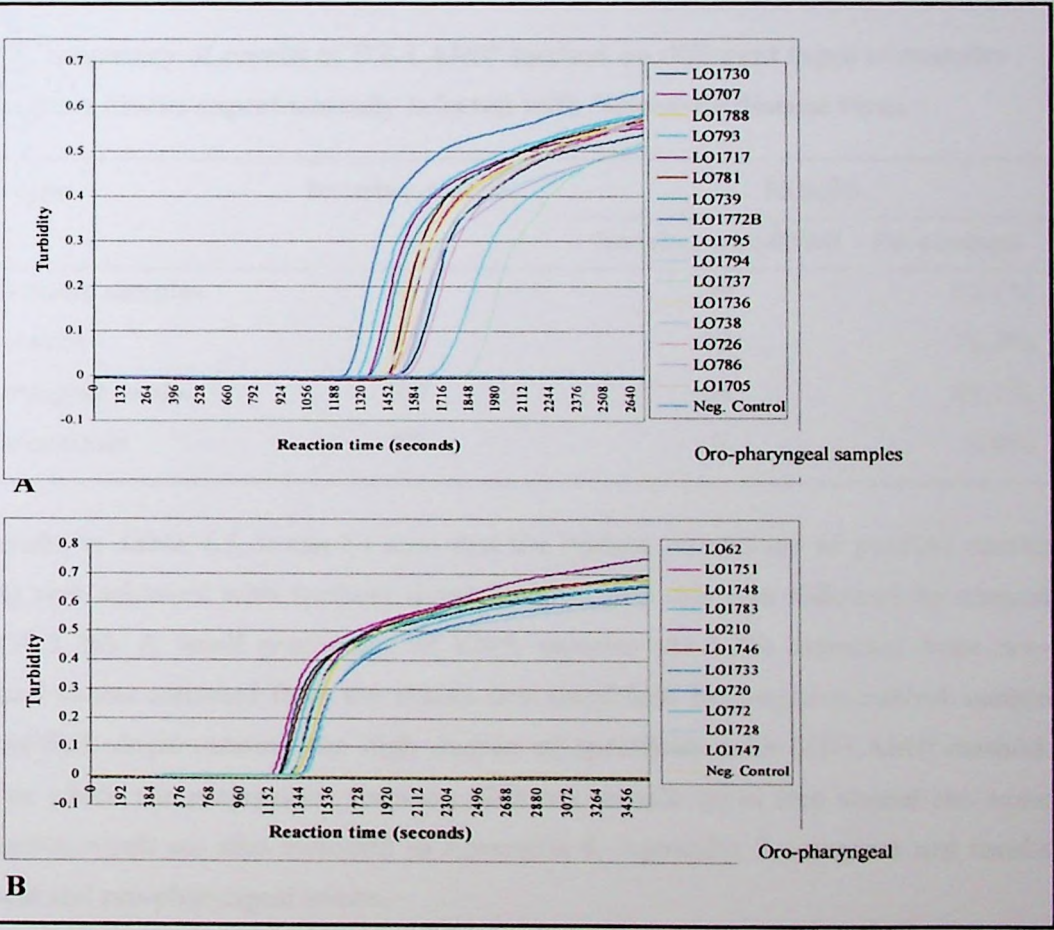


Figure 4.8. RT-LAMP reaction (A and B batches) during amplification of RNA extracted from oro-pharyngeal swabs

From the results shown in the two batches in the figure and those not shown, the analysis by real-time turbidimetry indicated that Newcastle disease viral RNA of 44 (88 %) samples was amplified (tested positive) and six samples (12 %) were negative by RT-LAMP. In the two batches (A and B) analyzed using all the negative control samples were test- negative by the method.

**4.3.6 A summary of Viral RNA detection by RT-LAMP method**

The results of RT-LAMP by heat block and real-time turbidimetry for tracheal tissue samples, and cloacal and oro-pharyngeal swabs from chicks experimentally infected with ND virus were compiled. A summary of these results is presented in Table 4.5.

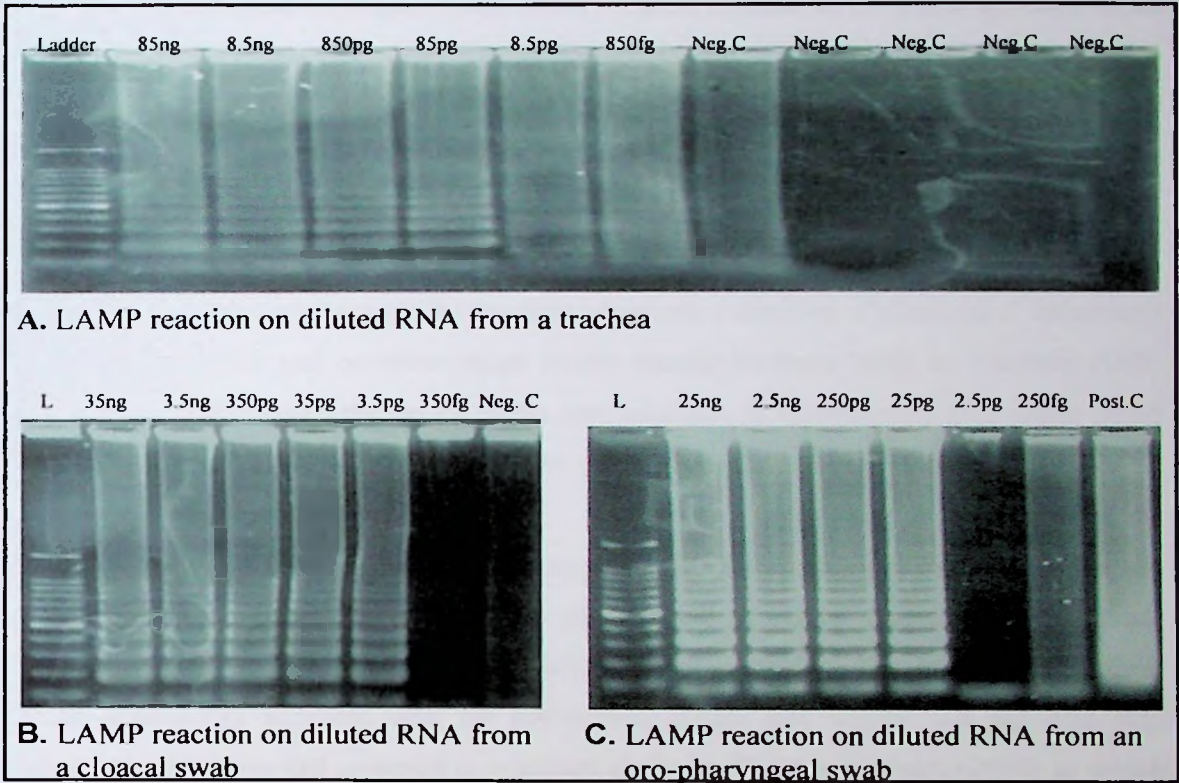
**Table 4.5. Summary of results of RT-LAMP method on different types of samples collected from chicks experimentally infected with Newcastle disease virus**

| Sample type             | Number tested | Results          |            |
|-------------------------|---------------|------------------|------------|
|                         |               | Number amplified | Percentage |
| Tracheal tissue samples | 41            | 39               | 95.1%      |
| Cloacal swabs           | 92            | 72               | 78.3%      |
| Oro-pharyngeal swabs    | 107           | 44               | 41.1%      |
| Negative controls       | 07            | 0                | 0.0%       |

From results in Table 4.5, it can be seen that the highest percentage of positive results (95.1 %) was achieved with tracheal tissue samples and this was followed by cloacal swabs (78.3 %). A small percentage of RNA samples (41.1 %) extracted from oro-pharyngeal swabs collected from the chicks was amplified. No negative control sample was amplified, demonstrating the high degree of specificity with RT-LAMP method. Details in which the proportions showing different sample types that shared the same amplification result are also indicated in Appendix 6. Appendix 7 compares test results for cloacal and oro-pharyngeal swabs.

**4.3.7 Analytical sensitivity of RT-LAMP test in the detection of Newcastle disease virus using non-tissue samples**

For determination of analytical (diagnostic) sensitivity of the primer set used in this study (Uga2), the amount of RNA in different samples, that is, tracheal tissue, cloacal swab and oro-pharyngeal swab, were first determined as 85 ng, 35 ng and 25 ng, respectively.



**Figure 4.9. Sensitivity of LAMP primers on a serially diluted positive RNA extracted from samples collected by different methods from chickens infected with Newcastle disease virus**

Figure 4.9 shows the preliminary single test detection limits by RT-LAMP using serially diluted Newcastle disease viral RNA from the three categories of samples. As shown by smears in the multiple bands of the agarose gel in the figure, the detection limits of the method are demonstrated.

In the test, tracheal tissue samples had a detection limit of 850 fg (0.85 pg), followed by cloacal sample (3.5 pg); oro-pharyngeal samples had the highest (25 pg). The detection limit of 0.85 pg achieved in this study was only slightly higher than that reported in a

related study by Pham and others (2005). The analytical sensitivity for ND viral RNA from the trachea specimens (tissue samples), as reported in that study was 0.5 pg.

#### 4.4 Discussion

RT-LAMP method achieved a ND viral RNA detection rate of 95.1 % with tracheal tissue samples (Table 4.5), and within 19:30 – 36:00 minutes at 64°C and agrees with an earlier study by Pham and others (2005). Detection rate of 78.3 % and 41.1 % were also achieved with cloacal swabs and oro-pharyngeal swabs, respectively by RT-LAMP method. Much as the result indicated a fairly high detection rate with cloacal swabs the detection by the method on oro-pharyngeal swabs, as specimens for sample collection, demonstrates poor virus recovery. The probable cause for the low virus recovery rate in the oro-pharyngeal swabs could be the time of sample collection. Collection of specimens in form of cloacal and oro-pharyngeal swabs should be done early in infection (OIE, 2007). The collection of the swabs at the time of death or immediately after death could have affected the virus recovery rate by the oro-pharyngeal swabs.

Since no negative sample was amplified by the method, this provides evidence of the test property of high specificity reported of this method in earlier studies (Notomi *et al.*, 2000; Mori *et al.*, 2001, Baba *et al.*, 2002; Pham *et al.* 2005). The low detection limit (high sensitivity) achieved with the preliminary single test results (0.8 pg) was only slightly higher than that reported in a related study by Pham and others (2005) in which the analytical sensitivity for Newcastle disease viral RNA from the trachea specimens (tissue samples) was reported as 0.5 pg. This provides additional evidence of the high level of analytical sensitivity of RT-LAMP in the detection of Newcastle disease viral RNA.

Since RT-LAMP method was able to detect ND virus infection with high analytical sensitivity, ease and within a short time, the Null hypothesis was upheld and hence state that RT-LAMP diagnostic technique can effectively (good sensitivity and specificity and efficiently (easy to use and fast) detect virus in non-tissue samples (cloacal and oro-pharyngeal swabs), as it can with tissue samples. Only the degree of amplification can be interpreted as fair for the cloacal swabs and poor for oro-pharyngeal swabs.

Optimization test of the primer set used in the study achieved RNA amplification at a reaction temperature of 64°C in a reaction time of 33:48 minutes. The primers, therefore, demonstrated good amplification efficiency for LAMP reactions. Good amplification efficiency should be achieved in about 30 minutes at temperature of 60°C - 65°C (Notomi *et al.*, 2000).

The decline of immunity (from 7Log2 to as low as 0Log2) of the chicks against ND is in agreement with the expected trend of decline of maternal antibodies among chicks. Maternal antibodies are undetectable in most chicks by 28 days post-hatch, though some chicks may remain protected as late as 42 days post hatch (Nemeth *et al.*, 2007). By the 3<sup>rd</sup> day of the 5<sup>th</sup> week during the study HI titres among the chicks had declined to almost 0Log2 in 62 % of the chicks sampled. This trend of decline provided the evidence that passive immunity, which could interfere with the infection post inoculation, had waned. Inoculation of chicks with NDV was, therefore, done at a time when infection could effectively be achieved. High levels of passive antibodies in young chicks produced from immunized parent flocks can interfere with replication of vaccine/active virus strains (Beard *et al.*, 1984).

The pathogenesis and morbidity and mortality rates observed among the chicks during the period of infection were similar to those described by McFerran *et al.* (1988). These were cardinal features in increasing the certainty that Newcastle disease virus, with which the chicks were inoculated, was the cause of the observed effects. The trend of the clinical picture observed with the chicks during the infection showed the active involvement stage of the disease (3-5 days post-inoculation with a virulent NDV infection). The virulent ND virus during day 3-5 post infection depresses dietary intake and hence rendering the chicks vulnerable to further pathological effects (Samsone *et al.* (1964). The morbidity rate of 100 % and mortality rate of >80 % has been reported in highly susceptible chickens infected with the velogenic strain of NDV (Alexander, 2003).

**4.5 Recommendations**

1. There is need to conduct more studies on the use of oro-pharyngeal or buccal swabs in the detection of Newcastle disease virus by RT-LAMP method.
2. More work should be done to determine the specificity of the LAMP primers used in this study.
3. More works is required to determine precision, repeatability and reproducibility of RT-LAMP on non-tissue samples

## **Chapter 5**

### **Assessment of the Potential Application of Reverse Transcriptase Loop-Mediated Isothermal Amplification for Diagnosis of Newcastle Disease in Free-Range Poultry**

#### **Abstract**

The potential of using Reverse Transcriptase Loop-mediated isothermal amplification (RT-LAMP) in the diagnosis of Newcastle disease (ND) in free-range diseased chickens under different epidemiological factors was assessed. Management system, flock size, age group, climatic conditions and the cost of analysis were considered during the study.

ND viral RNA extracted from 30 tracheal tissues, 67 cloacal swabs and 59 oro-pharyngeal swabs from clinical/dead cases of chickens naturally exposed to ND infection and confirmed by immunochromatographic assay was analysed. Samples from adult and growing chickens kept in flock sizes ranging from as low as 01-10 to as high as 71-80, from five sub-counties in two districts enjoying different climatic conditions in Uganda were collected. The samples were analysed by RT-LAMP and were compared with those obtained by immunocromatographic assay. The estimated cost for analysis of a sample by the method was computed and compared with that by RT-PCR. The epidemiological sensitivity and specificity were also determined. Data analysis was done by McNamara's test.

Of the 44 clinical cases from which samples were collected, 40 (90.9 %) were positive by chromatographic immunoassay, 100 % (n=44) of the tracheal tissues and cloacal swabs and 95.5 % (n=44) of the oro-pharyngeal swabs were test-positive by RT-LAMP. The epidemiologic sensitivity and specificity of RT-LAMP was 100 % and 100 % for tracheal tissues, 97 % and 100 % for cloacal swabs and 80 % and 100 % for the oro-pharyngeal swabs, respectively.

The majority of the samples (78.6 %) were amplified with in 19:30-36:00 minutes. The estimated cost of analyzing a single sample incubated in a turbidimeter, waterbath and

heat block was \$ 0.076, \$ 0.005 and \$ 0.004, respectively, compared to the cost (\$ 0.09) when RT-PCR is used (all exclusive of RNA extraction costs).

The detection of Newcastle disease virus infection by RT-LAMP using cloacal swabs could provide an easier, cheaper and faster molecular-based test for field diagnosis of the infection in free-range chickens in developing countries.

*Key words: Newcastle disease virus infection, RT-LAMP, free-range chicken, cloacal swab and oro-pharyngeal swabs.*

## 5.1 Introduction

Uganda has an estimated population of 33.0 million poultry 80% of which is reared in the traditional free-range systems in the villages (Mukiibi, 1992; FAO, 2006). The flocks are largely small size (5–30 chickens), mixed age, distributed in many households and feed mainly by scavenging (Awan *et al.*, 1994). In Uganda farming activities are geographically divided into seven agro ecological zones. These zones have got different climatic conditions. Among these agro-ecological zones are the Banana-coffee farming system and the Banana-millet-cotton farming system. Much as temperatures in the two zones show little variation (25°C – 31°C) throughout the year, the Banana-coffee system receives evenly distributed rainfall amounting to 1,000 mm – 1,500 mm and the Banana-millet-cotton receives the same amount of but less stable rainfall (Mwebaze, 2006). These conditions influence the management style and welfare of chickens. In these agro-ecological zones chicken rearing is practiced in over 88 % of the households. The chicken management method has been free-range. But recently, semi-intensive management has been adopted by some farmers.

Newcastle disease (ND) is the most important infectious disease affecting free-range village chickens, particularly in developing countries including Uganda (Aini *et al.*, 1989; Mukiibi, 1992 and NARO, 1996). The disease is a highly pathogenic and contagious virus infection of poultry causing severe economic and production losses worldwide (Alexander 1988a). ND epidemics in developing countries result in great socio-economic losses and affect human health due to reduced food availability (Spadbrow, 1990; Martin, 1992; Alexander, 2003). Many reports and studies (Bell and Moloudi, 1988; Bell *et al.*, 1990; McBride *et al.*, 1991) suggest a continuous presence of Newcastle Disease Virus (NDV) in village poultry populations. The usual source of infection is assumed to be the other chickens (Spadbrow, 1990). Survival of chickens against ND is longer (>100 days) in the dry season compared to the rainy season (Otim *et al.*, 2004).

Field diagnosis of ND in Uganda is currently based mainly on history, clinical presentation and postmortem lesions. Laboratory diagnosis is commonly done by means

of Haemagglutination test, virus isolation and Haemagglutination inhibition (HI) test. HI is currently regarded the gold standard in the diagnosis of the disease (OIE, 2004). Another recently developed test for rapid diagnosis of ND is chromatographic immunoassay (Anigen, 2005). All these tests have got disadvantages of mainly having low specificity or low sensitivity. Apart from HA test which can be used early in infection, all the rest are used at least four days of exposure to viral infection.

Recently a novel nucleic acid amplification method termed Loop-mediated isothermal Amplification (LAMP) was developed (Notomi *et al.*, 2000). The characteristics of LAMP are rapidity under isothermal conditions, low reaction temperature (63°C to 65°C), and high specificity for the target sequence (Notomi *et al.*, 2000 and Mori *et al.*, 2001). Results of a study by Pham and others (2005) specifically demonstrated that the LAMP-based assay is a useful tool in the rapid and sensitive diagnosis of NDV and that all LAMP-positive samples in their study were also positive by nested PCR.

Since LAMP method has demonstrated a high analytical sensitivity (0.5 pg) and specificity (100 %), rapidity and ease in the detection of ND virus infection in experimental infections (Pham *et al.*, 2005) and as shown in Chapter 4 of this dissertation, the potential use of a convenient and rapid test like this one is necessary (IAEA/FAO, 2006) for field diagnosis of ND viral infection. Under usual circumstances, when a new test for disease is developed, its results are often compared to those from the existing standard, yet imperfect test (gold standard). If a gold standard is not available, it is only possible to assess the agreement between different tests, without assuming that one test is the best. Therefore, in circumstances where the sensitivity and specificity of a test to be evaluated is required and yet such a test is biologically similar to the standard test(s), its results can be compared with those of such a test(s) in order to determine the two properties for the new test (Martin *et al.*, 1987). For this purpose, animals positive to all tests in the test bank are assumed diseased and animals that are negative to all the tests in the bank assumed to be disease-free (Martin *et al.*, 198; Mateu-de-Antonio *et al.*, 1993). However, in such a case the results of the comparisons should be prefixed with relative, to indicate that the determinations were based on biologically related tests (Martin *et al.*, 1987).

There are a number of ways to evaluate a diagnostic test, varying from technical studies of calibration to controlled trials of the effect upon patient outcome (Martin *et al.*, 1987). However, evaluation of a diagnostic test usually refers to measurement of the diagnostic parameters of the test: sensitivity, specificity, positive and negative predictive values, and likelihood ratios (Martin *et al.*, 1987). The sensitivity of a diagnostic method is the proportion of true positive that are detected by the method while the specificity of the method on the other hand, is the proportion of true negatives that are detected (Martin *et al.*, 1987). The sensitivity and specificity of a diagnostic test are important in deciding the value of the test in disease control campaigns and in categorizing animals in observational studies (Thrusfield, 2001). Sensitivity and specificity can be quoted either as a probability between 0 and 1, or as a percentage (Thrusfield, 2001). These parameters can be calculated for tests in which the variable measured is nominal or dichotomous, for example, presence or absence of antigen (Thrusfield, 2001). Once a diagnostic technique measures what it purports to measure, it is valid (Thrusfield, 2001). Generally, at sensitivity and specificity of 99 % and 99 %, respectively, a test can be regarded relatively good for epidemiological studies (Martin *et al.*, 1987).

Therefore, this study sought to assess the possibility of using RT-LAMP in the diagnosis of ND viral infections under the selected factors under which free-range village chickens are commonly managed in Uganda. The time and cost implications of using RT-LAMP were also assessed.

## **5.2 Materials and methods**

### **5.2.1 Source of samples**

The chickens used in the study were from flocks reported in the outbreaks of Newcastle disease in the districts of Iganga and Tororo (Appendix 11). These districts lie within different Agro-ecological zones; Iganga district lies in the Banana-coffee farming system and Tororo lies in the Banana-millet-cotton farming system. In the two agro-ecological zones chicken rearing is practiced in over 88 % of the households. The sub-counties of Ibulanku, Makutu and Bulamagi in Iganga district and Mukuju and Osukuru, in Tororo district were the specific areas of the study.

### **5.2.2 Type of samples used in the study**

Various types of samples, which included tracheal tissues, cloacal and oro-pharyngeal swabs were collected during the study. Chromatographic immunoassay (*Anigen test*) was used to confirm NDV infection in chickens before collection of samples from a given flock. The flocks from which the samples were collected were of different sizes, mixed age groups (chicks, growers and adults chickens) and under different management systems (free-range and semi-intensive), but all of local chickens. Collection of samples was done for six months in two seasons; three months in the rainy season and three months in the dry season.

### **5.2.3 Sample size determination**

The sample size was determined based on Bachmann (2006) in whose study it was revealed that the median sample size for studies on diagnostic accuracy was 117. Also considered was McNamara's test requires at least 20 matched samples to be used. Therefore, a total of 156 samples were used in this study. They included 30 tracheal tissues, 67 cloacal swabs, 59 oro-pharyngeal swabs and four negative controls. Flocks from which samples were taken were selected based on confirmation of the clinical signs. The chickens or carcasses from which samples were collected were randomly selected.

### **5.2.4 Procedures used during RNA extraction from samples**

Ribonucleic Acid (RNA) from the tracheal tissues and cloacal and oro-pharyngeal swabs was extracted by use of the QUAamp® Viral RNA Mini Kit (Quagen, 2007). The materials, reagents and procedures are as described in Chapter 3.

### **5.2.5 Procedures of RT-LAMP Analysis**

The NDV RNA extracted from the 156 samples was analyzed by RT-LAMP. The set of the six optimized primers (four LAMP primers and two Loop primers) used in the experimental study (Chapter 4) was used for the amplification of RNA. The RNA Amplification Kit and the LAMP test procedures described in Chapter 3 were used in this study.

5.2.6 Measuring sensitivity and specificity of RT-LAMP method

The sensitivity and specificity of RT-LAMP in detecting NDV in non-tissue samples were obtained in two different comparisons. The first comparison was made between RT-LAMP using non-tissue samples (new test) and immunochromatographic assay (gold standard). The second comparison was done between this new test (RT-LAMP on non-tissue samples) and RT-LAMP on tissue samples (old test). The method described by Martin *et al.* (1987) was employed as illustrated below (Table 5.1).

Table 5.1. Layout of evaluation of sensitivity and specificity of a test

|                                                                    | Old test (Gold standard) |                    |                    |               |
|--------------------------------------------------------------------|--------------------------|--------------------|--------------------|---------------|
|                                                                    |                          | Test positive (T+) | Test negative (T-) | Total         |
| New test (RT-LAMP for ND using cloacal and oro-pharyngeal samples) | Test positive (T+)       | a (true positive)  | b (false negative) | (a+b)         |
|                                                                    | Test negative (T-)       | c (false positive) | d (true negative)  | (c+d)         |
|                                                                    | Total                    | (a+c)              | (b+d)              | (a+b) + (c+d) |

The sensitivity and specificity of RT-LAMP assay on the diagnosis of Newcastle disease in non-tissue samples were calculated. The sensitivity (Se), which is the proportion of the truly diseased birds that test positive, was calculated using the formula below:

Sensitivity (Se) =  $\frac{a}{a+c}$  [ that is,  $P(T^+/D^+)$  ]

The specificity (Sp), which is the proportion of the non-diseased birds that test negative, was also calculated. The following formula was used:

Specificity (Sp) =  $\frac{d}{b+d}$  [ that is,  $P(T^-/D^-)$  ]

5.2.7 Measuring agreement of RT-LAMP assay on non-tissue samples

Since the RT-LAMP test results are categorical (dichotomous), a kappa statistic was used to measure the level of agreement between the set of results for immunochromatographic assay or RT-LAMP on tissue samples and the results of RT-LAMP on the non-tissue samples. This was done by, first summing up the specific test results in a 2 x 2 contingency table. The calculations were done to determine the level of agreement (*kappa*) between the test results of the old and new test in each category of comparison. A qualitative assessment of kappa suggests that if it is high (>0.6), the tests are measuring what they purport to measure. If kappa is low (<0.2), much uncertainty exists and in the absence of sensitivity and specificity it is difficult to say which test provides the more valid answers (Martin *et al.*, 1987) . The method described by Martin *et al.* (1987) was used and the data layout for assessing agreement shown in Table 5.2. The assessment was done independent of each other using the same set of outcome categories.

Table 5.2. Layout for comparing results from two qualitative (dichotomous) tests

|          |                 | Old Test (Gold standard) |                 | Total           |
|----------|-----------------|--------------------------|-----------------|-----------------|
|          |                 | Test 2 positive          | Test 2 negative |                 |
| New Test | Test 1 positive | n <sub>11</sub>          | n <sub>12</sub> | n <sub>1.</sub> |
|          | Test 1 negative | n <sub>21</sub>          | n <sub>22</sub> | n <sub>2.</sub> |
|          |                 | n <sub>.1</sub>          | n <sub>.2</sub> | n               |

Using the results in the table the statistic called Cohen’s kappa was calculated. The essential elements of kappa were easily calculated as below:

Observed agreement = (n<sub>11</sub>+ n<sub>22</sub>)/n

Expected agreement (chance) = {[ (n<sub>1.</sub> \* n<sub>.1</sub>) / n ] + [ (n<sub>2.</sub> \* n<sub>.2</sub>) / n ] / n }

Actual agreement beyond chance = Observed agreement – expected agreement

Potential agreement beyond chance = (1 – expected agreement)

Kappa agreement beyond chance = Actual agreement beyond chance/Potential

### 5.2.8 Data analysis

Before calculating kappa, an assessment of whether there was a test bias was done. This was indicated by the proportion positive to each test differing (that is  $P^1 \neq P^2$ , that is, level of discordance, where  $P^1$  and  $P^2$  represent the proportion of positive to tests 1 and 2, respectively). Because the data was paired (data collected and recorded was based on whether or not the set of samples collected from each subject tested positive or negative to each of the two tests under comparison - nominal data), presence of a test bias was assessed by non-parametric statistics. The appropriate statistical test, therefore, was McNamara's test. McNamara's test is the appropriate test in studies involving comparing two sets of data from the same subjects under different test diagnostic tests (circumstances) (Motulsky, 1995).

During the analysis we let:

$P^1$  = Probability of a positive result using immunochromatographic assay or RT-LAMP assay on tissue samples.

$P^2$  = Probability of a positive result using RT-LAMP assay on non-tissue samples.

Then the test of the Null hypothesis was done as follows:

$H_0: P^1 = P^2$

$H_a: P^1 \neq P^2$

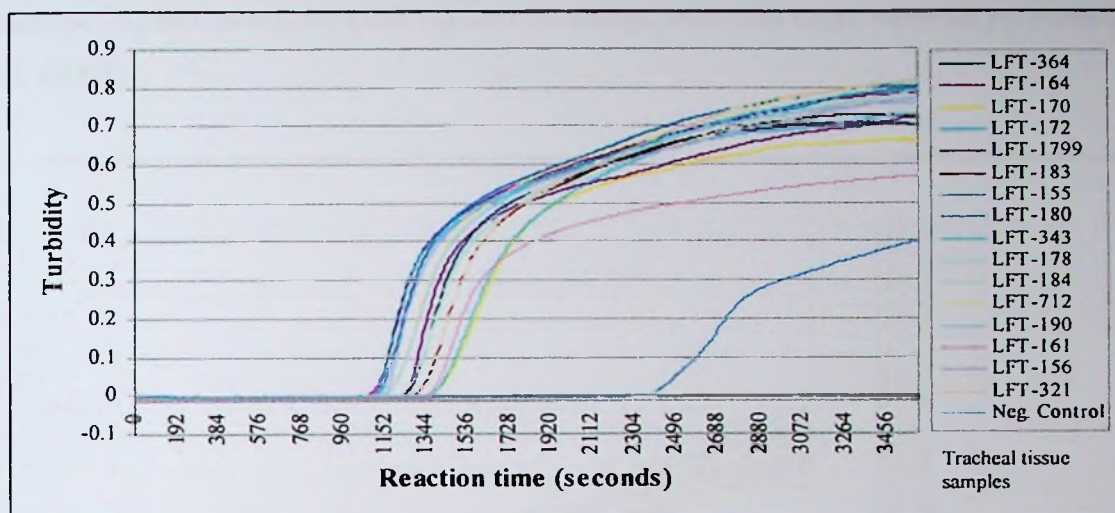
Test Statistic:  $M = (n_{12} - n_{21})^2 / (n_{12} + n_{21})$

Critical Region: Reject  $H_0$  in favour of  $H_a$  at 5 % if  $M \geq 3.84$

## 5.3 Results

### 5.3.1 RT-LAMP reaction using tracheal tissue samples

Ribonucleic acid (RNA) extracted from tracheal tissues was analysed by RT-LAMP using two methods. Fourteen samples were incubated in a heat block and results observed by UV-light and the naked eye. All (100 %) samples incubated by this method (results not shown) were positive by RT-LAMP. The other tracheal samples ( $n=16$ ) analysed by RT-LAMP method using real-time turbidimetry. The analysis was conducted in one batch. Figure 5.1 shows the results of RNA amplified using loopamp real-time turbidimeter. In this analysis all the 16 tracheal tissue samples were amplified.



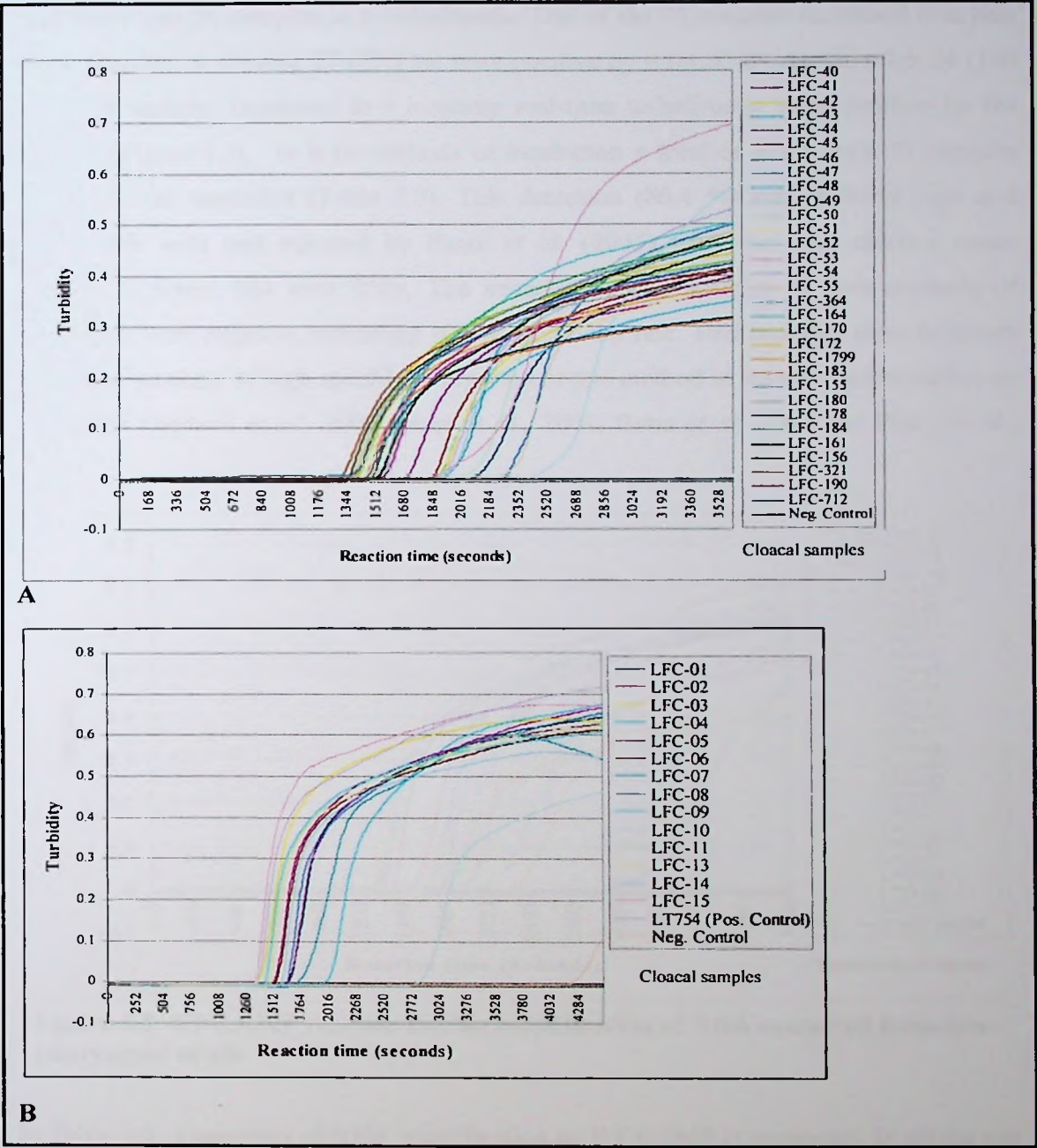
**Figure 5.1. RT-LAMP reaction during amplification of RNA extracted from tracheal samples**

The results demonstrated a high level (100 %) of detection by RT-LAMP, in case the ND viral RNA used is extracted from tracheal tissue samples. This high rate of detection of RT-LAMP method on tracheal tissue samples was also reported in an earlier study by Pham and others (2005).

### 5.3.2 RT-LAMP reaction using cloacal swabs

Ribonucleic acid (RNA) extracted from 67 cloacal samples collected from chickens naturally exposed to the velogenic strain of Newcastle disease virus were analysed by RT-LAMP method. These samples were incubated by two methods; 22 samples in a heat block and 45 samples in a turbidimeter. All the 22 samples incubated in a heat block (results not shown), were positive by RT-LAMP. Of the other 45 RNA samples incubated in a loopamp real-time turbidimeter in two batches, RNA from 43 samples (95.6 %) was amplified (Figure 5.2). In both methods of incubation a total of RNA from 65 samples was amplified. This indicates a detection rate of 97 % for RT-LAMP on cloacal swabs (Table 5.3). The one negative control sample used in the heat block and the two used in the two batches of analysis by turbidimetry tested negative to RT-LAMP. The results of the amplified samples demonstrate a fairly high rate of RNA detection, which is comparable with the rate of detection obtained with the tissue samples. This detection

rate (97 %) and specificity (100 %) are also comparable with those reported by Pham *et al.* (2005).



**Figure 5.2. RT-LAMP reaction (A and B batches) during amplification of RNA extracted from cloacal swabs**

5.3.3 RT-LAMP reaction using oro-pharyngeal swabs

Ribonucleic acid (RNA) extracted from 59 oro-pharyngeal swab samples were analysed by RT-LAMP method. These samples were incubated by two methods; 35 samples in a heat block and 24 samples in a turbidimeter. Out of the 35 samples incubated in a heat block (results not shown), 27 (77.1 %) were positive by RT-LAMP. All the other 24 (100 %) RNA samples incubated in a loopamp real-time turbidimeter were positive by the method (Figure 5.3). In both methods of incubation a total of RNA from 51 samples (86.4 %) was amplified (Table 5.3). This detection (86.4 %) rate is fairly high and comparable with that reported by Pham *et al.* (2005) when he used chicken tissue samples to detect ND viral RNA. The negative control samples in both methods of incubation were negative, indicating 100 % specificity rate. This provides more evidence of the test property of high specificity reported of this method in several studies earlier on conducted (Notomi *et al.*, 2000; Mori *et al.*, 2001, Baba *et al.*, 2002 and Pham *et al.*, 2005).

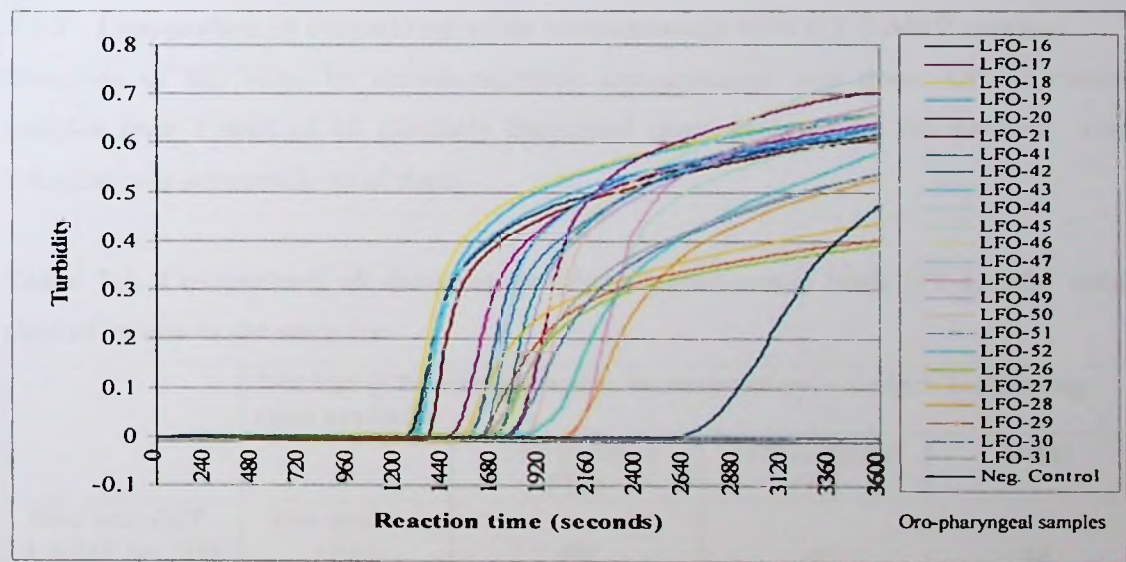


Figure 5.3. RT-LAMP reaction during amplification of RNA extracted from oro-pharyngeal swabs

In Table 5.3, a summary of RNA amplification by RT-LAMP is presented. In all the test reactions involving use of RNA from the tracheal samples, the tests were positive. The detection rate for NDV using the method on cloacal swabs was quite high (97 %). The

detection rate of 86.4 % for RNA from oro-pharyngeal swabs fairly high if compared with that obtained with samples collected from experimental chicks.

**Table 5.3. Summary of results of RT-LAMP on different types of samples**

| Sample type              | Number tested | Results          |            |
|--------------------------|---------------|------------------|------------|
|                          |               | Number amplified | Percentage |
| Tracheal tissue samples  | 30            | 30               | 100.0 %    |
| Cloacal swabs            | 67            | 65               | 97.0 %     |
| Oro-pharyngeal swabs     | 59            | 51               | 86.4 %     |
| Negative control samples | 4             | 0                | 0.0%       |

Details on the proportions of sample types that shared the same amplification results are indicated in Appendix 8.

**5.3.4 Comparison of chromatographic immunoassay with RT-LAMP method**

Detection of ND virus by chromatographic immunoassay was done. Of the cloacal samples from a total of 44 clinically diagnosed cases analysed by the method, viral infection was detected in 40 of them.

**Table 5.4. Comparison of chromatographic immunoassay with RT-LAMP using cloacal swabs as the samples**

|                                         | Old test (Chromatographic immunoassay – <i>Anigen</i> test) using cloacal swabs |                    |                    |       |
|-----------------------------------------|---------------------------------------------------------------------------------|--------------------|--------------------|-------|
|                                         |                                                                                 | Test positive (T+) | Test negative (T-) | Total |
| New test (RT-LAMP for ND cloacal swabs) | Test positive (T+)                                                              | 40                 | 4                  | 44    |
|                                         | Test negative (T-)                                                              | 0                  | 2                  | 2     |
|                                         | Total                                                                           | 40                 | 6                  | 46    |

The test did not detect viral infection in all the two non-diseased birds used in this part of the study. RNA extracted from the same samples (cases) was positive by RT-LAMP

analysis in all the 44 (100 %) clinically diagnosed cases. All (100 %) the two non-diseased cases were test-negative by RT-LAMP. Using the results of Table 5.4, the sensitivity (Se) and specificity (Sp) of RT-LAMP assay in the diagnosis of Newcastle disease was calculated. Chromatographic immunoassay (Anigen ®) was considered the old test (Gold standard) against whose results those of RT-LAMP method (new test) were compared.

The diagnostic properties (sensitivity and specificity) were calculated as follows:

$$\text{Sensitivity (Se)} = \frac{a}{(a+c)} = \frac{40}{40+0} = 1.0$$

$$\text{Specificity (Sp)} = \frac{d}{(b+d)} = \frac{2}{(4+2)} = 0.33$$

Determination of the level of agreement between the two tests:

An assessment of the existence of test bias was first made. This would be indicated by the proportion positive to each test differing (that is,  $P^1 \neq P^2$ ).

Where;  $P^1$  = Probability of a positive result using chromatographic immunoassay.

$P^2$  = Probability of a positive result using LAMP assay on non-tissue samples.

Being tested is;  $H_0: P^1 = P^2$

$H_a: P^1 \neq P^2$

Test Statistic:  $M = (n_{12} - n_{21})^2 / (n_{12} + n_{21})$

Critical Region: Reject  $H_0$  in favour of  $H_a$  at 5 % if  $M \geq 3.84$

$$\begin{aligned} \text{Therefore, McNemer's test (M)} &= \frac{(n_{12} - n_{21})^2}{(n_{12} + n_{21})} = \frac{(4 - 0)^2}{(4 + 0)} \\ &= \frac{4^2}{4} = \frac{16}{4} = 4 \end{aligned}$$

Since  $4 > 3.84$  at 5 %, we therefore do not reject the  $H_0$  and state that there is a test bias in the diagnosis of Newcastle disease by RT-LAMP technique using cloacal swabs in

comparison with immunochromatographic assay using similar samples. In this case RT-LAMP is a better method than immunochromatographic assay.

This indicates that there is test bias. Therefore, assessment of level of agreement is of little value.

**Table 5.5. Comparison of chromatographic immunoassay with RT-LAMP using oro-pharyngeal swabs as the samples**

|                                                | Old test (Chromatographic immunoassay – <i>Anigen</i> test) using cloacal swabs |                    |                    |       |
|------------------------------------------------|---------------------------------------------------------------------------------|--------------------|--------------------|-------|
|                                                |                                                                                 | Test positive (T+) | Test negative (T-) | Total |
| New test (RT-LAMP for ND oro-pharyngeal swabs) | Test positive (T+)                                                              | 40                 | 2                  | 42    |
|                                                | Test negative (T-)                                                              | 0                  | 4                  | 4     |
|                                                | Total                                                                           | 40                 | 6                  | 46    |

Using the results of this table, the sensitivity (Se) of LAMP assay in the diagnosis of Newcastle disease can be calculated as follows:

$$\text{Sensitivity (Se)} = \frac{a}{(a+c)} = \frac{40}{40 + 0} = 1.0$$

The specificity (Sp), on the other hand can also be calculated as below:

$$\text{Specificity (Sp)} = \frac{d}{(b+d)} = \frac{2}{(2 + 4)} = 0.33$$

Determination of the level of agreement between the two tests:

An assessment of the existence of test bias was first made. This would be indicated by the proportion positive to each test differing (that is,  $P^1 \neq P^2$ ).

Where;  $P^1$  = Probability of a positive result using chromatographic immunoassay.  
 $P^2$  = Probability of a positive result using LAMP assay on non-tissue samples.

Being tested is;  $H_0: P^1 = P^2$

$H_a: P^1 \neq P^2$

Test Statistic:  $M = (n_{12} - n_{21})^2 / (n_{12} + n_{21})$

Critical Region: Reject  $H_0$  in favour of  $H_a$  at 5 % if  $M \geq 3.84$

$$\begin{aligned}\text{Therefore, McNemer's test (M)} &= \frac{(n_{12} - n_{21})^2}{(n_{12} + n_{21})} = \frac{(2 - 0)^2}{(2 + 0)} \\ &= \frac{2^2}{2} = \frac{4}{2} = 2\end{aligned}$$

Since  $2 < 3.84$  at 5 %, we therefore accept the  $H_0$  and state that there is no test bias in the diagnosis of Newcastle disease by RT-LAMP technique using oro-pharyngeal swabs in comparison with immunochromatographic assay using similar samples.

Since there is no bias, therefore, assessment of level of agreement is of value. The level of agreement, kappa, was calculated as below:

$$\text{Observed agreement} = ((n_{11} + n_{22})/n) = (40 + 4)/46 = 0.96$$

$$\begin{aligned}\text{Expected agreement (chance)} &= [(n_{1.} \cdot n_{.1})/n + (n_{2.} \cdot n_{.2})/n]/n \\ &= [(42 \cdot 40)/46 + (2 \cdot 6)/46]/46 = 0.77\end{aligned}$$

$$\begin{aligned}\text{Actual agreement beyond chance} &= \text{Observed agreement} - \text{expected agreement} \\ &= 0.96 - 0.77 = 0.19\end{aligned}$$

$$\begin{aligned}\text{Potential agreement beyond chance} &= (1 - \text{Expected agreement}) \\ &= 1 - 0.77 = 0.23\end{aligned}$$

$$\begin{aligned}Kappa &= \text{Actual agreement beyond chance/Potential agreement beyond chance} \\ &= 0.19/0.23 = 0.82\end{aligned}$$

Thus, the point estimate of *Kappa*, 0.82, suggests excellent agreement between the two methods in diagnosing Newcastle disease in chickens.

**5.3.5 Comparison of RT-LAMP on tissue samples with RT-LAMP on non-tissue samples**

RT-LAMP reaction results for tracheal tissue samples and the results of cloacal swabs were compiled in a 2X2 table similar to Table 5.4 and Table 5.5 The sensitivity and specificity of RT-LAMP using cloacal swabs were calculated. The level of agreement (Kappa) and McNamara’s test were calculated, too. The decision about the existence of test biasness was taken. The very procedure was used for RT-LAMP using oro-pharyngeal swabs. Table 5.6 presents the summary results of the diagnostic properties of RT-LAMP in the diagnosis of Newcastle disease using cloacal swabs and oro-pharyngeal swabs, as separate tests.

**Table 5.6. Summary of sensitivity and specificity of RT-LAMP in the diagnosis of Newcastle disease using either cloacal or oro-pharyngeal swabs**

| Source of samples   | Sample category      | Test Sensitivity | Test Specificity | McNemer’s test | Conclusion         | Kappa ( $\kappa$ )           |
|---------------------|----------------------|------------------|------------------|----------------|--------------------|------------------------------|
| Free-range chickens | Cloacal swabs        | 0.97             | 1.0              | 1.0            | No test bias       | Substantial agreement (0.79) |
|                     | Oro-pharyngeal swabs | 0.80             | 1.0              | 6              | A test bias exists | -                            |

The results presented in the table indicate that RT-LAMP method using cloacal swabs for diagnosis of Newcastle disease infection achieved an epidemiological sensitivity and specificity of 0.97 and 1.0, respectively. Much as the sensitivity of the method lower than that reported by Pham and others (2005), the sensitivity reported in this study is fair and its specificity is perfect (Thrusfield, 2001). The level of agreement with the pre-existing RT-LAMP (0.79) is very high.

**5.3.6 Assessment of RT-LAMP analysis under different epidemiological factors**

The assessment made to evaluate the ability of RT-LAMP in the detection of ND viral RNA from samples collected from chickens being kept under different factors is presented in Table 5.7. The results in Table 5.9 indicate that all (100 %) the tracheal tissue samples were amplified by RT-LAMP. This was regardless of the sub-county, season of sample collection, the management system under which the exposed chickens

were being kept or the age group from which the sample was collected (Table 5.7 and Appendix 9 and 10). The amplification of ND viral RNA extracted from cloacal swabs collected from the different areas of study was also achieved by the method. The proportion of the cloacal swabs amplified however varied.

**Table 5.7. Distribution of RT-LAMP reaction positive samples by agro-ecological zone, management system, age group and season**

| Test                                                               | Factor of assessment |                             | Geographical location |                    |                   |                  |                   |
|--------------------------------------------------------------------|----------------------|-----------------------------|-----------------------|--------------------|-------------------|------------------|-------------------|
|                                                                    |                      |                             | Iganga district       |                    |                   | Tororo district  |                   |
|                                                                    |                      |                             | Ibulanku<br>(n=69)    | Bulamagi<br>(n=33) | Makuutu<br>(n=18) | Mukuju<br>(n=12) | Osukuru<br>(n=12) |
| Percentage<br>of samples<br>amplified<br>by RT-<br>LAMP<br>(n=156) | Type of<br>samples   | Tracheal<br>tissues         | 100.0%                | 100.0%             | 100.0%            | 100.0%           | 100.0%            |
|                                                                    |                      | Cloacal<br>swabs            | 100.0%                | 83.3%              | 100.0%            | 92.3%            | 100.0%            |
|                                                                    |                      | Oro-<br>pharyngeal<br>swabs | 84.0%                 | 75.0%              | 83.3%             | 100.0%           | 100.0%            |
|                                                                    | Season               | Rainy season                | 94.2%                 | 90.9%              | -                 | -                | -                 |
|                                                                    |                      | Dry season                  | -                     | -                  | 92.3%             | 97.1%            | 100.0%            |
|                                                                    | Mgt<br>system        | Free-range                  | 95.3%                 | 92.9%              | 92.3%             | 95.7%            | 100.0%            |
|                                                                    |                      | Semi-<br>intensive          | 88.9%                 | 60.0%              | -                 | 100.0%           | 100.0%            |
|                                                                    | Age<br>group         | Adults                      | 93.3%                 | 84.6%              | 88.9%             | 94.1%            | 100.0%            |
|                                                                    |                      | Growers                     | 90.0%                 | 100.0%             | 100.0%            | 100.0%           | 100.0%            |

The lowest percentage (83.3 %) was with the sub-county of Bulamagi, followed by Mukuju sub-county. All the cloacal samples collected from the other three sub-counties, regardless of the district of location tested positive. This demonstrated the ability of RT-LAMP to detect ND virus infection from samples collected from chickens infected under different agro-ecological zones. Oro-pharyngeal samples from the different sub-counties were amplified in this study. The percentages of the positive samples were 75.0 % for Bulamagi sub-county, 83.3 % for Makuutu and 84.0 % for Ibulanku sub-county. All (100 %) oro-pharyngeal samples from the other two sub-counties were all amplified. The results, together, demonstrate that ability of RT-LAMP to amplify ND viral RNA

extracted from oro-pharyngeal swabs is possible but only less efficient. This suggests that oro-pharyngeal swabs could be a poor specimen for virus recovery from the oropharynx. The results in the table also show that RT-LAMP can detect ND virus from samples collected from local chickens of all ages on free-range or semi-intensive management system exposed to ND virus infection during a rainy or dry season (Table 5.7 and Appendices 11 and 12).

The other factor on which RT-LAMP method was assessed was detection of ND virus infection among chickens in different flock sizes. Table 5.8 shows the results of the proportion (%) of the samples amplified and the different flock sizes from which such samples were collected.

**Table 5.8. Proportions of RT-LAMP reaction positive samples of chickens kept in different flock sizes**

| Flock Size (n=156) | Tracheal Tissues | Cloacal Swabs | Oro-pharyngeal Swabs |
|--------------------|------------------|---------------|----------------------|
| 01-10              | 100.00%          | 100.00%       | 100.00%              |
| 11-20              | 100.00%          | 96.80%        | 89.30%               |
| 21-30              | 100.00%          | 100.00%       | 75.00%               |
| 31-40              | 100.00%          | 100.00%       | 75.00%               |
| 41-50              | 100.00%          | 100.00%       | 100.00%              |
| 51-60              | 100.00%          | 66.70%        | 75.00%               |
| 61-70              | 100.00%          | 100.00%       | 100.00%              |
| 71-80              | 100.00%          | 100.00%       | 100.00%              |

As shown in the table, all the tracheal tissue samples collected from the chickens kept in different flock sizes were amplified. The amplification reaction results on cloacal was 100 % for samples from all flock sizes apart from the flock sizes of 11-20 and 51-60 chickens in which the percentage of amplified samples are 96.8 % and 66.7 %, respectively. The proportion of oro-pharyngeal test-positive samples was lowest (75.0 %) with the flock sizes of 21-30, 31-40 and 51-60. This was followed by 89.3 % of the amplified oro-pharyngeal samples in the size of 11-20 chickens. In the rest of the flock

sizes all the oro-pharyngeal samples were amplified. This gives an indication that all the three types of sample were amplified but tracheal samples are better amplified followed by the cloacal swabs. The amplification of oro-pharyngeal swabs is interpreted to be poor by this study.

### **5.3.7 The cost, ease and time (rapidity) implications of using RT-LAMP in the diagnosis of Newcastle disease in free-range poultry.**

The estimated cost of using RT-LAMP for the detection of ND viral RNA was calculated. This was, however, based on LAMP reagents that are currently used for research purposes only. The estimated costs of RT-LAMP in this case were compared with the cost of analyzing the same category of sample by RT-PCR, the test with which they share similar biological/analytical properties. Unlike RT-PCR where the only equipment used (and considered in this case) is a thermocycler, in RT-LAMP the consideration of a real-time turbidimeter, waterbath and heat block were considered as alternative equipment in incubation of reaction mixtures.

Since the cost of the primers is almost similar for the two tests, the calculation of the cost for the purpose of determining the cost of analyzing a single sample was not considered. This cost is not included in the final cost of analyzing a sample by any of the two methods presented in Table 4.9.

In order to provide a realistic estimate of the cost of analyzing one sample by each of the two methods, it was assumed that the equipment's useful life be taken as five years. Also assumed is that the number of samples whose analysis uses this equipment per day is 50. The number of days the analysis is done, using the equipment, per month was taken to be 22 working days (excluding weekends). The RNA QUAamp® Viral RNA Mini Kit for 50 samples (elutions) taken at a cost of \$ 400 was used as a basis for calculating extraction cost.

Using simple arithmetic, therefore: the total number of samples analysed using each of the scientific equipment =

50 samples per day x 22 days per month x 12 months in a year x 5 years = 66,000 samples.

Based on the above assumptions and current market prices of the equipment and reagents, the computed cost of analyzing one sample by each of the two methods is contained in Table 5.9.

**Table 5.9. Cost estimation for analyzing one sample by RT-PCR/RT-LAMP**

| Test    | Equipment used | Estimated equipment cost |             | Estimated cost for RNA extraction of one sample |         | Estimated cost of analyzing one sample |         | Estimated cost of analyzing one sample (less extraction cost) |      |
|---------|----------------|--------------------------|-------------|-------------------------------------------------|---------|----------------------------------------|---------|---------------------------------------------------------------|------|
|         |                | USD                      | UShs        | USD                                             | UShs    | USD                                    | UShs    | USD                                                           | UShs |
| RT-PCR  | Thermocycler   | 6,000                    | 10,320,000= | 8                                               | 13,760= | 8.090                                  | 13,915= | 0.090                                                         | 155= |
| RT-LAMP | Turbidimeter   | 5,000                    | 8,600,000=  | 8                                               | 13,760= | 8.076                                  | 13,891= | 0.076                                                         | 131= |
|         | Waterbath      | 332                      | 571,040=    | 8                                               | 13,760= | 8.005                                  | 13,769= | 0.005                                                         | 9=   |
|         | Heat Block     | 284                      | 488,480=    | 8                                               | 13,760= | 8.004                                  | 13,767= | 0.004                                                         | 7=   |

Currency exchange rate = \$1 = Uganda shillings: 1720

As seen in table 5.9, the cost of analyzing a sample by RT-PCR is \$ 8.090 (Ushs 13915) inclusive of extraction costs and \$ 0.090 (Ushs 155) exclusive of extraction costs. This is a higher cost than that of RT-LAMP by real-time turbidimetry, which is \$ 8.076 (Ushs 13,891) inclusive of extraction cost or \$ 0.076 (Ushs 131) exclusive of extraction costs. The costs are much lower for RT-LAMP where the waterbath and heat block are used. The costs in this case are estimated at \$ 8.005 (13,769)/ \$ 0.005 (Ushs 9) and \$ 8.004 (13,761)/\$ 0.004 (Ushs 7), with/without consideration of extraction costs, respectively.

In the study it was realized that the RT-LAMP products could be easily seen with a naked eye based on the developed turbidity achieved with a single reaction temperature of incubation. This made the interpretation of the RT-LAMP results very simple; no special skills required.

In 75.3 % of the reactions (n=295) conducted by real-time RT-LAMP amplification was achieved at  $\leq 35$  minutes reaction time with the lowest time of 19:30 minutes. Only two test reactions went beyond 45 minutes. This reaction time was far lower than that taken by nested RT-PCR (six hours) or real time RT-PCR (1½ hours).

#### 5.4 Discussion

In this study, detection rates of 97.0 % and 86.4 % using cloacal swabs and oro-pharyngeal swabs were achieved. The detection rate of RT-LAMP method with cloacal samples is higher than that of the method where oro-pharyngeal swabs are used as the samples. This probably indicates that the amount of virus and the rate of shedding are higher with the cloacal route than the respiratory one. Cloacal swabs can, therefore, be taken as a better specimen in ND viral recovery by RT-LAMP than the oro-pharyngeal swabs. The tracheal tissue samples however remain superior of the three sample types in this method. The results of this study are in agreement with those reported by Pham and others (2005).

The high specificity (1.0) for all the negative control samples was additional evidence of the degree specificity reported of this method in several other studies (Notomi *et al.*, 2000; Mori *et al.*, 2001, Baba *et al.*, 2002 and Pham and others (2005). The epidemiologic sensitivity and specificity achieved for RT-LAMP on cloacal swabs, of 97 % and 100 % are good, respectively. Generally, at sensitivity and specificity of 99 % and 99 %, respectively, a test can be regarded good for epidemiological studies (Martin *et al.*, 1987).

The epidemiologic sensitivity of 100 % achieved with RT-LAMP method on cloacal and oro-pharyngeal swabs as compared with immunochromatographic assay in this study, is good. This sensitivity indicates that RT-LAMP can be used in the detection of proportion of true positive in flocks exposed to Newcastle disease infection (Martin *et al.*, 1987).

The results of cloacal swabs, as analyzed by RT-LAMP and immunochromatographic assay, indicate existence of test biasness between the two methods. In this bias RT-

LAMP is regarded a better method in diagnosis of Newcastle disease virus infection using cloacal swabs in comparison with immunochromatographic assay. Much as the number of samples for each of cloacal and oro-pharyngeal swabs is only 44 and 42, respectively, the two put together provide sample size beyond the smallest (71) ever used in studies on determination of diagnostic accuracy of tests (Bachmann *et al.*, 2006).

Despite the difference in the factors under which the chickens of sample collection were being kept, the samples collected from the clinical cases in the different conditions did not affect their analysis results by RT-LAMP. The samples from the chickens in the three sub-counties in Iganga district were collected during the rainy season and the results of samples analysis were not different from those of samples from the two sub-counties of Tororo District from which samples were collected during the dry season. This, therefore, suggests that rainfall and probably temperature did not have any effect on virus recovery from samples collected during the two seasons of the year and the different agro-ecological zones of the Uganda.

Since the samples were from clinical cases in the age range of both growers and adult chickens and no difference was noted in the results of their analysis by RT-LAMP. It is possible that age related factors did not affect virus recovery by oro-pharyngeal and cloacal swabs.

The results by RT-LAMP analysis of samples from clinical cases of chickens from different flock sizes did not show any variation that could be attributed to flock size. No difference was also attributed to the management system of free-range or semi-intensive system. Virus recovery in swabs from clinical cases, seem not to be affected by management system or flock sizes in chicken management.

The estimated cost of analyzing a single sample by RT-LAMP method was found lower (\$8.004) than analyzing it by RT-PCR (\$ 8.090), especially if a heat block is used. This cost could be made much lower if the extraction costs can be minimized; analyzing a sample by RT-LAMP exclusive of RNA extraction costs can be as low as \$ 0.004 or \$

0.005, if the reaction mixture is incubated by a heat block or waterbath, respectively. This, combined with the fact that the reaction time of the test is 19:30-35:00 minutes and results interpreted with a naked eye, makes the use of RT-LAMP method applicable in cheap, fast and effective field laboratories in developing countries.

## Chapter 6

### 6.0

### General Discussion

In this study, RT-LAMP method achieved a Newcastle disease viral RNA detection rate of 95.1 % with tracheal tissue samples (from experimentally infected chicks) (Table 4.4). A detection rate (100 %) of the viral RNA was obtained with the similar type of tissue samples collected from the chickens naturally exposed to the velogenic strain of ND virus in field outbreaks (Table 5.3). This result provides additional evidence of the ability of RT-LAMP in the detection of ND virus from tracheal tissue samples as earlier reported by Pham and others (2005).

In this study detection rates of 78.3 % and 41.1 % were achieved by RT-LAMP on cloacal swabs and oro-pharyngeal swabs, respectively, from experimental infection. Also achieved are the rates of 97 % and 86.4 % using cloacal swabs and oro-pharyngeal swabs from naturally exposed chickens. The detection rates with cloacal samples are fairly high in both cases. This detection rate of Newcastle disease viral RNA extracted from cloacal swabs, which is close to rates reported by Pham and others (2005), demonstrates that this type of material is good specimens for virus recovery and can be used in LAMP studies. The high specificity (100 %) is similar to reports by other workers (Notomi *et al.*, 2000; Mori *et al.*, 2001; Baba *et al.*, 2002; Pham *et al.*, 2005).

The detection rate of ND virus in oro-pharyngeal swab samples collected from the experimental chicks using RT-LAMP technique, was however very low (41.1 %). Similarly, when samples from natural exposure were analysed, oro-pharyngeal swabs showed the lowest virus detection rate (86.4 %) compared to cloacal swabs (97.0 %). The probable cause for the low virus recovery rate in the oro-pharyngeal swabs could be the time of sample collection. Collection of specimens in form of cloacal and oro-pharyngeal swabs should be done early in infection (OIE, 2007). The collection of the swabs at the time of death or immediately after death could have affected the virus recovery rate by the oro-pharyngeal swabs.

The lowest detection limit achieved with the preliminary single test results (Figure 4.9) in this study was 0.8 pg. This limit was only slightly higher than that reported in a related study by Pham and others (2005) of 0.5 pg. This provides additional evidence of the high level of analytical sensitivity of RT-LAMP in the detection of Newcastle disease viral RNA.

The epidemiologic sensitivity and specificity achieved for RT-LAMP on cloacal swabs, of 0.97 and 1.0, respectively, achieved with the natural exposure cases in this study is fairly good for epidemiological studies. No epidemiological studies for evaluation of RT-LAMP in Newcastle disease have been reported before. Therefore, no comparison can be made in this case (Martin *et al.*, 1987).

Despite the difference in the factors under which the chickens were living at the time of exposure and samples collection, the samples from the clinical cases in the different conditions did not affect their analysis results by RT-LAMP. The samples from the chickens in the three sub-counties in Iganga district were collected during the rainy season and the results obtained when the samples were analyzed were not different from those of samples from the two sub-counties of Tororo District whose samples were collected from the dry season. This, therefore, suggests that there rainfall and probably temperature did not have any effect on virus recovery from such samples. There was no difference attributed to age group, management system or flock size.

The estimated cost of analyzing a single sample by RT-LAMP method was found lower (\$8.076) than analyzing it by RT-PCR (\$ 8.09), especially if a heat block is used. This cost could even be made much lower if the extraction costs can be minimized; analyzing a sample by RT-LAMP with consideration of RNA extraction costs can be as low as \$ 0.004 or \$ 0.005, if the reaction mixture is incubated using a heat block or waterbath, respectively.

Optimization test of the primer set used in the study was able to achieve RNA amplification at a reaction temperature of 64°C in 33:48 minutes. The primers, therefore, demonstrated good amplification efficiency as indicated for LAMP reactions. Good

amplification efficiency is said to be achieved in about 30 minutes at a reaction temperature of 60°C - 65°C (Notomi *et al.*, 2000).

The decline of immunity (from 7Log2 to as low as 0Log2) in the chicks against (Table 4.1) is in agreement with the expected trend of decline of maternal antibodies among chicks from a protected parent stock. Maternal antibodies are undetectable in most chicks by 28 days post-hatch, but some chicks remained protected as late as 42 days post hatch (Nemeth *et al.*, 2007). By the 3<sup>rd</sup> day of the 5<sup>th</sup> week the highest HI titre among experimental chicks was 1Log2. The titres of 62 % of the chicks sampled in the flock were as low as 0Log2. The progress of decline had to be monitored in order to ensure that inoculation of chicks with NDV is done at a time when infection could effectively be achieved. High levels of passive antibodies in young chicks produced from immunized parent flocks can interfere with replication of vaccine/active virus strains (Beard *et al.*, 1984).

The pathogenesis and the morbidity and mortality rates observed among the chicks during the disease were similar to those described by McFerran and others (1988). These were cardinal features in increasing the certainty of infection, with among chicks inoculated with the ND virus. The trend of the clinical picture observed with the chicks during the infection showed the active involvement stage of the disease 3-5 days post-inoculation with a virulent NDV. The virulent Newcastle disease virus during day 3-5 post infection depresses dietary intake and hence rendering the chicks vulnerable to further pathological effects (Samsone *et al.*, 1964). The morbidity rate of 100 % and mortality rate of >80 % increased the certainty that the clinical signs were due to NDV infection.

## Chapter 7

### 7.0

### Conclusions and Recommendations

#### 7.1 Conclusions

1. This study has demonstrated that RT-LAMP method can amplify Newcastle disease viral RNA with fairly good epidemiological sensitivity (89 %) and specificity (80 %) when cloacal swabs are used as the samples during exposure to Newcastle disease. The method detected Newcastle disease viral RNA with good analytical test sensitivity (0.8 pg) when cloacal swabs (non-tissue samples) were used. The estimated cost of using RT-LAMP method are fairly low (\$8.005 – 8.004), especially if the waterbath or heat block are used for incubation of reaction mixtures. The reaction time can be as fast as 19:30-35 minutes at an isothermal temperature (64°C).
2. RT-LAMP method demonstrated a fair and good epidemiologic sensitivity (97 %) and specificity (100 %), respectively, when cloacal swabs were used as samples in the detection of Newcastle disease virus from field outbreaks.
3. RT-LAMP method in the diagnosis of Newcastle disease when cloacal swabs collected from infected chickens are used has varying levels of agreement, kappa, when compared with the test diagnosis using tracheal tissue samples. A moderate level of agreement (0.57) was realized with sample collected from chicks and a substantial level of agreement (0.79) with samples collected from adult chickens in free-range poultry. Therefore, RT-LAMP can be used, along with other tests, in the field diagnosis of Newcastle disease in free-range poultry.
4. Since no other study has published findings on epidemiologic sensitivity and specificity of RT-LAMP in the diagnostic of Newcastle disease, results of this study, therefore, provide a basis for future work.
5. The findings of this study are interpreted in favour of the Null hypothesis and therefore stated that RT-LAMP diagnostic technique can effectively (good sensitivity and specificity) and efficiently (easy to use, cost effective and is fast) detect

Newcastle disease virus from cloacal swabs collected from infected chickens as it can, with the tissue samples. The Null hypothesis that chickens, on free-range type of management, with clinical Newcastle disease, as a result of natural exposure to the causative virus, can be identified by RT-LAMP analysis is also maintained (upheld).

## **7.2 Recommendations**

1. As earlier on recommended that tracheas and lungs (tissue samples) are good samples for detection of NDV in the diagnosis of Newcastle disease with high sensitivity and specificity, cloacal swabs (non-tissue samples) be adopted for the field diagnosis of ND in chickens. RT-LAMP method's laboratory sensitivity and the epidemiologic sensitivity and specificity are fairly good and hence RT-LAMP be adopted as a rapid and inexpensive test for field use.
2. More studies should be done in the use of oro-pharyngeal swabs in the detection of Newcastle disease virus by RT-LAMP assay.
3. There is need to conduct more studies to determine the precision, reproducibility and repeatability of RT-LAMP in the diagnosis of Newcastle disease using cloacal swabs.
4. Work should be done to determine the level of agreement among the various methods (turbidimetry, UV-light, naked eye and gels) of visualizing LAMP products.
5. There is need to develop effective and efficient but cheap reagents that help reduce the extraction costs in order that analysis by molecular-based techniques can be made cheaper.
6. A more comprehensive study be undertaken to assess the diagnostic performance of RT-LAMP in all aspects that affect the survival chicken and the Newcastle disease virus as a pathogen.

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## Appendix 1

### Preparation of reagents used in HA/HI tests

#### A. Preparation of Acid Citrate Dextrose (ACD)

- |                      |         |
|----------------------|---------|
| 1. Citric acid       | 4.0 g   |
| 2. Trisodium citrate | 11.26 g |
| 3. D-glucose         | 11.0 g  |

- Make up to 500 ml with de-ionized water.
- Sterilize by autoclaving at 115°C for 10 minutes.

#### B. Preparation of Dextrose Gelatine Veronal (DGV)

For 1 litre;

- |                                                           |         |
|-----------------------------------------------------------|---------|
| 1. Barbitone                                              | 0.58 g  |
| 2. Gelatine                                               | 0.60 g  |
| 3. Barbitone sodium                                       | 0.30 g  |
| 4. <b>Either</b> $\text{CaCl}_2 \cdot \text{H}_2\text{O}$ | 0.026 g |
| <b>OR</b> $\text{CaCl}_2$ anhydrous                       | 0.02 g  |
| 5. $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$              | 0.12 g  |
| 6. NaCl                                                   | 8.50 g  |
| 7. D-glucose                                              | 10.0 g  |

- Dissolve Barbitone and Gelatine in 125 ml of de-ionized water with the aid of a little heat.
- Add this to the other reagents dissolved in 600 ml of de-ionized water (pH 7.0 – 7.6)
- Make up to 1 litre and pour it in universal bottles 10 x 150 ml.
- Autoclave or pressure air-oven for 10 minutes at 101b.
- Store at 4°C.

## Appendix 2

**Reaction of differently designed primers on RNA extracted from a positive tracheal tissue sample collected from a Newcastle disease virus infected chicken**

| No. | Primer set | Tube No | Block & position on<br>Turbidimeter | LAMP Reaction<br>Temperature | LAMP<br>reaction |
|-----|------------|---------|-------------------------------------|------------------------------|------------------|
| 1   | Uga1       | 1       | B1                                  | 63°C                         | -                |
|     |            | 2       | B2                                  | 63°C                         | -                |
|     |            | 3       | D1                                  | 65°C                         | -                |
|     |            | 4       | D2                                  | 65°C                         | -                |
| 2   | Uga2       | 5       | C1                                  | 64°C                         | +                |
|     |            | 6       | C2                                  | 64°C                         | +                |
|     |            | 7       | D3                                  | 65°C                         | +                |
|     |            | 8       | D4                                  | 65°C                         | +                |
| 3   | Uga3       | 9       | A1                                  | 61°C                         | -                |
|     |            | 10      | A2                                  | 61°C                         | -                |
|     |            | 11      | B3                                  | 63°C                         | -                |
|     |            | 12      | B4                                  | 63°C                         | -                |

+ LAMP reaction and - Negative LAMP reaction

### Appendix 3

Results of RT-LAMP on RNA extracted from samples collected from each case of chicken by different methods

| No. | Chicken No. | RT-LAMP results as interpreted by the naked eye |              |                     |
|-----|-------------|-------------------------------------------------|--------------|---------------------|
|     |             | Tracheal tissue                                 | Cloacal swab | Oro-pharyngeal swab |
| 1   | 1732        | N/A                                             | N/A          | -                   |
| 2   | 1771        | N/A                                             | N/A          | -                   |
| 3   | 327         | +                                               | N/A          | N/A                 |
| 4   | 792         | N/A                                             | N/A          | -                   |
| 5   | 759         | +                                               | N/A          | N/A                 |
| 6   | 795         | N/A                                             | N/A          | -                   |
| 7   | 1753        | N/A                                             | N/A          | -                   |
| 8   | 1788        | +                                               | N/A          | N/A                 |
| 9   | 1709        | N/A                                             | N/A          | -                   |
| 10  | 1786        | N/A                                             | N/A          | -                   |
| 11  | 737         | N/A                                             | N/A          | -                   |
| 12  | 259         | N/A                                             | N/A          | -                   |
| 13  | 1732        | +                                               | N/A          | N/A                 |
| 14  | 787         | +                                               | N/A          | N/A                 |
| 15  | 1709        | +                                               | N/A          | N/A                 |
| 16  | 327         | N/A                                             | N/A          | -                   |
| 17  | 66          | N/A                                             | N/A          | -                   |
| 18  | 259         | +                                               | N/A          | N/A                 |
| 19  | 1758        | +                                               | N/A          | N/A                 |
| 20  | 1           | +                                               | N/A          | N/A                 |
| 21  | 2           | +                                               | N/A          | N/A                 |
| 22  | 3           | +                                               | N/A          | N/A                 |
| 23  | 4           | +                                               | N/A          | N/A                 |
| 24  | 5           | +                                               | N/A          | N/A                 |
| 25  | 6           | +                                               | N/A          | N/A                 |
| 26  | 7           | +                                               | N/A          | N/A                 |
| 27  | 8           | +                                               | N/A          | N/A                 |
| 28  | 9           | +                                               | N/A          | N/A                 |
| 29  | 10          | +                                               | N/A          | N/A                 |
| 30  | 11          | +                                               | N/A          | N/A                 |
| 31  | 13          | +                                               | N/A          | N/A                 |
| 32  | 14          | +                                               | N/A          | N/A                 |
| 33  | 15          | +                                               | N/A          | N/A                 |
| 34  | 7           | N/A                                             | N/A          | -                   |
| 35  | 8           | N/A                                             | N/A          | -                   |
| 36  | 9           | N/A                                             | N/A          | -                   |
| 37  | 10          | N/A                                             | N/A          | -                   |

|    |      |     |     |     |
|----|------|-----|-----|-----|
| 38 | 11   | N/A | N/A | -   |
| 39 | 15   | N/A | N/A | -   |
| 40 | 1757 | N/A | N/A | -   |
| 41 | 1758 | N/A | N/A | -   |
| 42 | 1712 | N/A | N/A | -   |
| 43 | 759  | N/A | N/A | -   |
| 44 | 798  | N/A | N/A | -   |
| 45 | 1785 | N/A | N/A | -   |
| 46 | 77   | N/A | N/A | -   |
| 47 | 63   | N/A | N/A | -   |
| 48 | 405  | N/A | N/A | -   |
| 49 | 71   | N/A | N/A | +   |
| 50 | 301  | N/A | N/A | +   |
| 51 | 93   | N/A | N/A | +   |
| 52 | 85   | N/A | N/A | +   |
| 53 | 96   | N/A | N/A | +   |
| 54 | 754  | N/A | N/A | -   |
| 55 | 787  | N/A | N/A | -   |
| 56 | 92   | N/A | N/A | -   |
| 57 | 94   | N/A | N/A | -   |
| 58 | 91   | N/A | N/A | -   |
| 59 | 408  | N/A | N/A | -   |
| 60 | 82   | N/A | N/A | -   |
| 61 | 58   | N/A | N/A | -   |
| 62 | 96   | N/A | -   | N/A |
| 63 | 66   | N/A | -   | N/A |
| 64 | 327  | N/A | -   | N/A |
| 65 | 91   | N/A | -   | N/A |
| 66 | 301  | N/A | -   | N/A |
| 67 | 1712 | N/A | -   | N/A |
| 68 | 94   | N/A | -   | N/A |
| 69 | 63   | N/A | -   | N/A |
| 70 | 62   | N/A | -   | N/A |
| 71 | 77   | N/A | -   | N/A |
| 72 | 93   | N/A | -   | N/A |
| 73 | 405  | N/A | -   | N/A |
| 74 | 408  | N/A | -   | N/A |
| 75 | 797  | N/A | -   | N/A |
| 76 | 92   | N/A | -   | N/A |
| 77 | 85   | N/A | -   | N/A |
| 78 | 1763 | N/A | N/A | -   |
| 79 | 789  | N/A | N/A | -   |
| 80 | 1773 | N/A | N/A | -   |
| 81 | 705  | N/A | N/A | -   |

|     |      |     |     |   |
|-----|------|-----|-----|---|
| 82  | 1789 | N/A | -   | - |
| 83  | 1729 | N/A | -   | - |
| 84  | 1797 | N/A | -   | - |
| 85  | 780  | N/A | -   | - |
| 86  | 1776 | N/A | -   | - |
| 87  | 713  | N/A | N/A | - |
| 88  | 1775 | N/A | N/A | - |
| 89  | 1782 | N/A | N/A | - |
| 90  | 1772 | N/A | N/A | - |
| 91  | 1769 | N/A | N/A | - |
| 92  | 54   | N/A | N/A | - |
| 93  | 1740 | N/A | N/A | - |
| 94  | 1784 | N/A | N/A | - |
| 95  | 1800 | N/A | N/A | - |
| 96  | 1749 | N/A | N/A | - |
| 87  | 729  | N/A | N/A | - |
| 88  | 712  | N/A | N/A | - |
| 89  | 1745 | N/A | N/A | - |
| 90  | 800  | N/A | N/A | - |
| 91  | 742  | N/A | N/A | - |
| 92  | 723  | N/A | N/A | - |
| 93  | 1718 | N/A | N/A | - |
| 94  | 727  | N/A | N/A | - |
| 95  | 1791 | N/A | N/A | - |
| 96  | 730  | N/A | N/A | - |
| 97  | 1759 | N/A | N/A | - |
| 98  | 1735 | N/A | N/A | - |
| 99  | 1724 | N/A | N/A | - |
| 100 | 1734 | N/A | N/A | - |
| 101 | 1799 | N/A | N/A | - |

+ = Positive sample; - = Negative sample; and N/A = sample not collected/analysis

#### Appendix 4

Results of RT-LAMP on sets of tracheas, cloacal and oro-pharyngeal samples each collected from the same experimentally infected chicken

| Chicken No. | Results of RT-LAMP on RNA extracted from samples collected from same chickens by different methods |              |                     |                 |
|-------------|----------------------------------------------------------------------------------------------------|--------------|---------------------|-----------------|
|             | Trachea                                                                                            | Cloacal swab | Oro-pharyngeal swab | Similar for all |
| 727         | +                                                                                                  | +            | -                   |                 |
| 729         | +                                                                                                  | +            | -                   |                 |
| 730         | +                                                                                                  | +            | -                   |                 |
| 737         | +                                                                                                  | +            | -                   |                 |
| 742         | +                                                                                                  | +            | -                   |                 |
| 759         | +                                                                                                  | +            | +                   | √               |
| 780         | +                                                                                                  | +            | -                   |                 |
| 787         | +                                                                                                  | +            | -                   |                 |
| 792         | +                                                                                                  | +            | -                   |                 |
| 795         | +                                                                                                  | +            | -                   |                 |
| 800         | -                                                                                                  | +            | -                   |                 |
| 1771        | +                                                                                                  | +            | -                   |                 |
| 1773        | +                                                                                                  | +            | -                   |                 |
| 1784        | +                                                                                                  | +            | -                   |                 |
| 1791        | +                                                                                                  | +            | -                   |                 |
| 1800        | +                                                                                                  | +            | -                   |                 |
| 259         | +                                                                                                  | -            | -                   |                 |
| 327         | +                                                                                                  | -            | -                   |                 |
| 66          | -                                                                                                  | -            | -                   | √               |
| 705         | +                                                                                                  | +            | -                   |                 |
| 71          | +                                                                                                  | +            | +                   | √               |
| 712         | +                                                                                                  | +            | -                   |                 |
| 713         | +                                                                                                  | +            | -                   |                 |
| 1709        | +                                                                                                  | +            | -                   |                 |
| 1712        | +                                                                                                  | -            | -                   |                 |
| 1718        | +                                                                                                  | +            | -                   |                 |
| 1729        | +                                                                                                  | +            | -                   |                 |
| 1731        | +                                                                                                  | -            | +                   |                 |
| 1732        | +                                                                                                  | +            | -                   |                 |
| 1734        | +                                                                                                  | +            | -                   |                 |
| 1740        | +                                                                                                  | +            | -                   |                 |
| 1745        | +                                                                                                  | +            | -                   |                 |
| 1751        | +                                                                                                  | +            | +                   | √               |
| 1753        | +                                                                                                  | +            | -                   |                 |
| 1758        | +                                                                                                  | +            | -                   |                 |
| 1763        | +                                                                                                  | +            | -                   |                 |
| 1769        | +                                                                                                  | +            | -                   |                 |
| Total (+)   | 35                                                                                                 | 32           | 04                  | 03              |
| % age (+)   | 94.59                                                                                              | 86.49        | 10.81               | 08.11           |

+ Positive sample; - Negative sample and √ similar result for all the three samples obtained from a given chicken

## Appendix 5

**Results of RT-LAMP on sets of tracheas, cloacal and oro-pharyngeal samples each collected from a given experimentally infected chick**

| Chicken No. | Results of RT-LAMP on RNA extracted from samples collected from same chickens by different methods |                     |                  |
|-------------|----------------------------------------------------------------------------------------------------|---------------------|------------------|
|             | Cloacal swab                                                                                       | Oro-pharyngeal swab | Similar for both |
| 727         | +                                                                                                  | -                   |                  |
| 729         | +                                                                                                  | -                   |                  |
| 730         | +                                                                                                  | -                   |                  |
| 737         | +                                                                                                  | -                   |                  |
| 738         | +                                                                                                  | +                   | √                |
| 739         | +                                                                                                  | +                   | √                |
| 742         | +                                                                                                  | -                   |                  |
| 746         | +                                                                                                  | +                   | √                |
| 759         | +                                                                                                  | +                   | √                |
| 77          | +                                                                                                  | -                   |                  |
| 772         | +                                                                                                  | +                   | √                |
| 780         | +                                                                                                  | -                   |                  |
| 786         | +                                                                                                  | +                   | √                |
| 787         | +                                                                                                  | -                   |                  |
| 789         | +                                                                                                  | -                   |                  |
| 792         | +                                                                                                  | -                   |                  |
| 795         | +                                                                                                  | -                   |                  |
| 797         | -                                                                                                  | +                   |                  |
| 798         | +                                                                                                  | -                   |                  |
| 800         | +                                                                                                  | -                   |                  |
| 82          | -                                                                                                  | -                   | √                |
| 85          | -                                                                                                  | +                   |                  |
| 91          | -                                                                                                  | -                   | √                |
| 92          | -                                                                                                  | -                   | √                |
| 93          | -                                                                                                  | +                   |                  |
| 94          | -                                                                                                  | -                   | √                |
| 96          | -                                                                                                  | +                   |                  |
| 1771        | +                                                                                                  | -                   |                  |
| 1773        | +                                                                                                  | -                   |                  |
| 1775        | +                                                                                                  | -                   |                  |
| 1776        | +                                                                                                  | -                   |                  |
| 1783        | +                                                                                                  | +                   | √                |
| 1784        | +                                                                                                  | -                   |                  |
| 1785        | +                                                                                                  | -                   |                  |
| 1788        | +                                                                                                  | +                   | √                |
| 1789        | +                                                                                                  | -                   |                  |
| 1791        | +                                                                                                  | -                   |                  |
| 1794        | +                                                                                                  | +                   | √                |
| 1795        | +                                                                                                  | +                   | √                |
| 1797        | +                                                                                                  | -                   |                  |
| 1800        | +                                                                                                  | -                   |                  |
| 210         | +                                                                                                  | +                   | √                |
| 259         | -                                                                                                  | -                   | √                |
| 301         | -                                                                                                  | +                   |                  |
| 327         | -                                                                                                  | -                   | √                |
| 405         | -                                                                                                  | -                   | √                |
| 408         | -                                                                                                  | -                   | √                |
| 62          | +                                                                                                  | +                   | √                |
| 63          | -                                                                                                  | -                   | √                |

|                                        |   |   |       |
|----------------------------------------|---|---|-------|
| 66                                     | - | - | √     |
| 705                                    | + | - |       |
| 707                                    | + | + | √     |
| 71                                     | + | + | √     |
| 712                                    | + | - |       |
| 713                                    | + | - |       |
| 720                                    | + | + | √     |
| 723                                    | + | - |       |
| 726                                    | + | + | √     |
| 1705                                   | + | - |       |
| 1709                                   | + | - |       |
| 1712                                   | - | - | √     |
| 1718                                   | + | - |       |
| 1724                                   | + | - |       |
| 1728                                   | + | + | √     |
| 1729                                   | + | - |       |
| 1730                                   | + | + | √     |
| 1731                                   | - | + |       |
| 1732                                   | + | - |       |
| 1733                                   | + | + | √     |
| 1734                                   | + | - |       |
| 1735                                   | + | - |       |
| 1736                                   | + | + | √     |
| 1737                                   | + | + | √     |
| 1738                                   | + | + | √     |
| 1739                                   | + | + | √     |
| 1740                                   | + | - |       |
| 1742                                   | + | + | √     |
| 1745                                   | + | - |       |
| 1746                                   | + | + | √     |
| 1747                                   | - | + |       |
| 1748                                   | + | + | √     |
| 1751                                   | + | + | √     |
| 1753                                   | + | - |       |
| 1758                                   | + | - |       |
| 1763                                   | + | - |       |
| 1769                                   | + | - |       |
| Number of similar results (n = 86)     |   |   | 38    |
| Percentage of similar results (n = 86) |   |   | 44.19 |

+ = Positive result; - = negative result; and √ = Positive samples in both cases

## Appendix 6

Comparing results of RT-LAMP on RNA extracted from different types of samples collected from experimentally infected chickens

| Factor                                                                                                       | (n) | Sample results |            |
|--------------------------------------------------------------------------------------------------------------|-----|----------------|------------|
|                                                                                                              |     | Number         | Percentage |
| Sets of samples (tracheas, cloacal and oro-pharyngeal swabs) with positive results for all                   | 37  | 03             | 08.11%     |
| Sets of samples (tracheas, cloacal and oro-pharyngeal swabs) with negative results for all                   | 37  | 01             | 02.70%     |
| Non-tissue samples (cloacal/oro-pharyngeal swabs) with results similar to those of tissue samples (tracheas) | 37  | 34             | 91.87%     |
| Sets of samples (cloacal and oro-pharyngeal swabs) with positive results for both.                           |     | 27             | 31.40%     |
| Sets of samples (cloacal and oro-pharyngeal swabs) with negative results for both.                           |     | 11             | 12.80%     |
| Tracheas samples with positive result                                                                        | 41  | 39             | 95.12%     |
| Tracheas samples with negative result                                                                        | 41  | 02             | 04.88%     |
| Cloacal swabs (samples) with positive results                                                                | 92  | 72             | 78.26%     |
| Cloacal swabs (samples) with negative results                                                                | 92  | 20             | 21.74%     |
| Oro-pharyngeal swabs (samples) with positive results                                                         | 107 | 44             | 41.12%     |
| Oro-pharyngeal swabs (samples) with negative results                                                         | 107 | 63             | 58.88%     |

n = Number of sets each of 3 samples

## Appendix 7

**Results of RT-LAMP on sets of tracheas, cloacal and oro-pharyngeal samples each collected from a given experimentally infected chick**

| Chicken No. | Results of RT-LAMP on RNA extracted from samples collected from same chickens by different methods |                     |                  |
|-------------|----------------------------------------------------------------------------------------------------|---------------------|------------------|
|             | Cloacal swab                                                                                       | Oro-pharyngeal swab | Similar for both |
| 738         | +                                                                                                  | +                   | √                |
| 739         | +                                                                                                  | +                   | √                |
| 746         | +                                                                                                  | +                   | √                |
| 759         | +                                                                                                  | +                   | √                |
| 772         | +                                                                                                  | +                   | √                |
| 786         | +                                                                                                  | +                   | √                |
| 82          | -                                                                                                  | -                   | √                |
| 91          | -                                                                                                  | -                   | √                |
| 92          | -                                                                                                  | -                   | √                |
| 94          | -                                                                                                  | -                   | √                |
| 1783        | +                                                                                                  | +                   | √                |
| 1788        | +                                                                                                  | +                   | √                |
| 1794        | +                                                                                                  | +                   | √                |
| 1795        | +                                                                                                  | +                   | √                |
| 210         | +                                                                                                  | +                   | √                |
| 259         | -                                                                                                  | -                   | √                |
| 327         | -                                                                                                  | -                   | √                |
| 405         | -                                                                                                  | -                   | √                |
| 408         | -                                                                                                  | -                   | √                |
| 62          | +                                                                                                  | +                   | √                |
| 63          | -                                                                                                  | -                   | √                |
| 66          | -                                                                                                  | -                   | √                |
| 707         | +                                                                                                  | +                   | √                |
| 71          | +                                                                                                  | +                   | √                |
| 720         | +                                                                                                  | +                   | √                |
| 726         | +                                                                                                  | +                   | √                |
| 1712        | -                                                                                                  | -                   | √                |
| 1728        | +                                                                                                  | +                   | √                |
| 1730        | +                                                                                                  | +                   | √                |
| 1733        | +                                                                                                  | +                   | √                |
| 1736        | +                                                                                                  | +                   | √                |
| 1737        | +                                                                                                  | +                   | √                |
| 1738        | +                                                                                                  | +                   | √                |
| 1739        | +                                                                                                  | +                   | √                |
| 1742        | +                                                                                                  | +                   | √                |
| 1746        | +                                                                                                  | +                   | √                |
| 1748        | +                                                                                                  | +                   | √                |
| 1751        | +                                                                                                  | +                   | √                |
|             |                                                                                                    |                     |                  |

## Appendix 8

Comparing results of RT-LAMP on RNA extracted from different types of samples collected from naturally infected chickens in the field

| Factor                                                                                                       | (n) | Sample results |            |
|--------------------------------------------------------------------------------------------------------------|-----|----------------|------------|
|                                                                                                              |     | Number         | Percentage |
| Sets of samples (tracheas and cloacal/oro-pharyngeal swabs) with positive results for both                   | 22  | 16             | 72.73%     |
| Sets of samples (tracheas, cloacal and oro-pharyngeal swabs) with negative results for all                   | 22  | 06             | 27.27%     |
| Non-tissue samples (cloacal/oro-pharyngeal swabs) with results similar to those of tissue samples (tracheas) | 30  | 29             | 96.67%     |
| Sets of samples (cloacal and oro-pharyngeal swabs) with positive results for both                            | 59  | 49             | 83.05%     |
| Sets of samples (cloacal and oro-pharyngeal swabs) with negative results for both                            | 59  | 0              | 0.00%      |
| Tracheas samples with positive result                                                                        | 30  | 30             | 100.0%     |
| Tracheas samples with negative result                                                                        | 30  | 0              | 00.00%     |
| Cloacal swabs (samples) with positive results                                                                | 67  | 65             | 97.01%     |
| Cloacal swabs (samples) with negative results                                                                | 67  | 02             | 2.99%      |
| Oro-pharyngeal swabs (samples) with positive results                                                         | 59  | 51             | 86.44%     |
| Oro-pharyngeal swabs (samples) with negative results                                                         | 59  | 08             | 13.56%     |

n = Number of sets of sample (each of three specimens)

## Appendix 9

### RT-LAMP reaction results of samples collected from chickens under different epidemiological factors

| Flock size | RT-LAMP reaction results |            |               |            |                      |            |
|------------|--------------------------|------------|---------------|------------|----------------------|------------|
|            | Tracheal tissues         |            | Cloacal swabs |            | Oro-pharyngeal swabs |            |
|            | Number                   | Percentage | Number        | Percentage | Number               | Percentage |
| 01-10      | 20                       | 100.0%     | 30            | 100.0%     | 20                   | 100.0%     |
| 11-20      | 16                       | 100.0%     | 31            | 96.8%      | 28                   | 89.3%      |
| 21-30      | 4                        | 100.0%     | 12            | 100.0%     | 8                    | 75.0%      |
| 31-40      | 1                        | 100.0%     | 2             | 100.0%     | 3                    | 75.0%      |
| 41-50      | 3                        | 100.0%     | 4             | 100.0%     | 3                    | 100.0%     |
| 51-60      | 3                        | 100.0%     | 3             | 66.7%      | 4                    | 75.0%      |
| 61-70      | 7                        | 100.0%     | 7             | 100.0%     | 7                    | 100.0%     |
| 71-80      | 2                        | 100.0%     | 2             | 100.0%     | 2                    | 100.0%     |

### RT-LAMP reaction results of samples collected from chickens in different seasons

| Season       | RT-LAMP reaction results |            |               |            |                      |            |
|--------------|--------------------------|------------|---------------|------------|----------------------|------------|
|              | Tracheal tissues         |            | Cloacal swabs |            | Oro-pharyngeal swabs |            |
|              | Number                   | Percentage | Number        | Percentage | Number               | Percentage |
| Rainy season | 11                       | 100.0%     | 40            | 97.5%      | 39                   | 82.1%      |
| Dry season   | 19                       | 100.0%     | 24            | 95.8%      | 20                   | 95.0%      |

### RT-LAMP reaction results of samples collected from chickens in different management systems

| Season         | RT-LAMP reaction results |            |               |            |                      |            |
|----------------|--------------------------|------------|---------------|------------|----------------------|------------|
|                | Tracheal tissues         |            | Cloacal swabs |            | Oro-pharyngeal swabs |            |
|                | Number                   | Percentage | Number        | Percentage | Number               | Percentage |
| Free-range     | 21                       | 100.0%     | 53            | 98.1%      | 49                   | 87.8%      |
| Semi-intensive | 09                       | 100.0%     | 12            | 91.7%      | 10                   | 80.0%      |

### RT-LAMP reaction results of samples collected from birds of different age groups

| Age group of chickens | RT-LAMP reaction results |            |               |            |                      |            |
|-----------------------|--------------------------|------------|---------------|------------|----------------------|------------|
|                       | Tracheal tissues         |            | Cloacal swabs |            | Oro-pharyngeal swabs |            |
|                       | Number                   | Percentage | Number        | Percentage | Number               | Percentage |
| Adults                | 16                       | 100.0%     | 33            | 93.9%      | 30                   | 90.0%      |
| Growers               | 14                       | 100.0%     | 32            | 100.0%     | 30                   | 86.7%      |

# Appendix 10

## RT-LAMP reaction results of samples collected from chickens in different households in the field

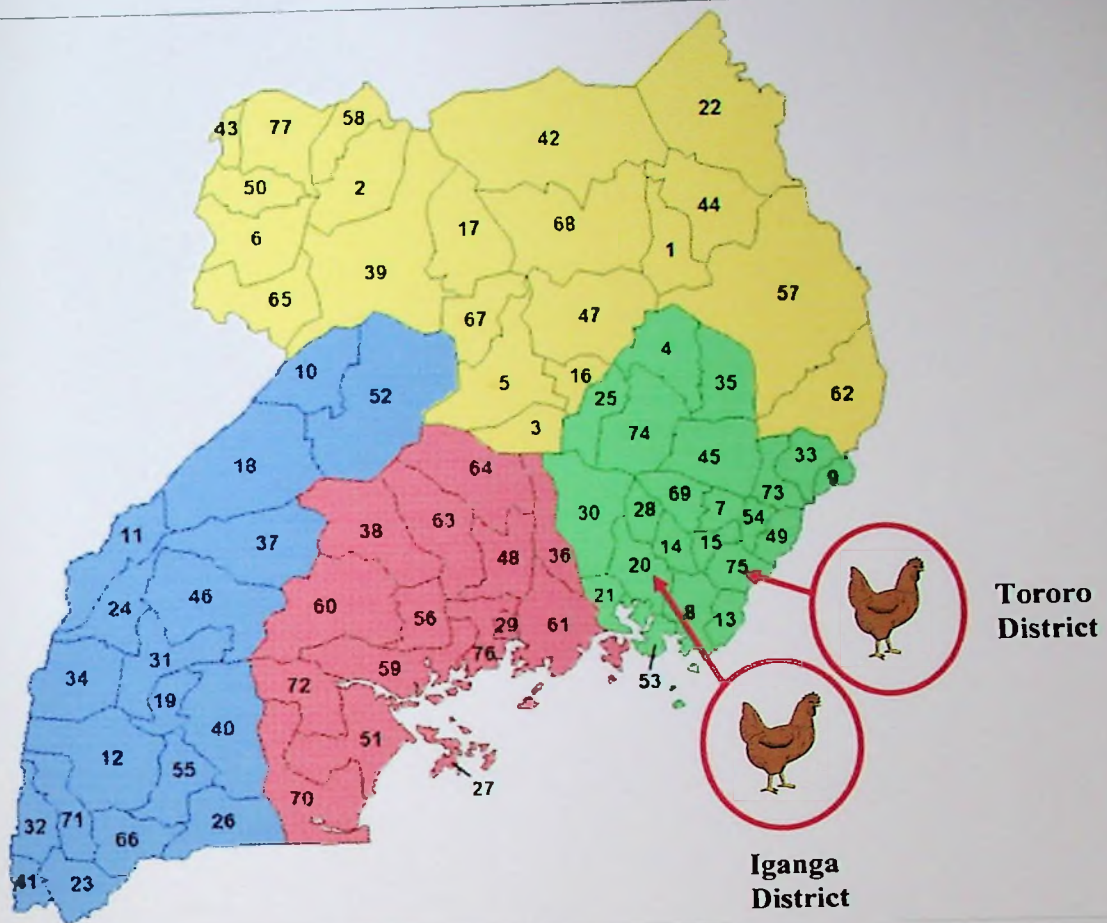
| District | Sub-county | No. | Household | Flock size | Mgt system     | Morbidity rate | Samples collected   | Result of sample from adults | Result of sample from growers |
|----------|------------|-----|-----------|------------|----------------|----------------|---------------------|------------------------------|-------------------------------|
| Iganga   | Ibulanku   | 1.  | II01      | 16         | Free-range     | 100.0%         | Tracheal tissue     | +                            | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | N/A                           |
|          |            | 2.  | II02      | 10         | Free-range     | 90.0%          | Oro-pharyngeal swab | N/A                          | +                             |
|          |            |     |           |            |                |                | Tracheal tissue     | +                            | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 3.  | II03      | 32         | Free-range     | 93.3%          | Oro-pharyngeal swab | +                            | N/A                           |
|          |            |     |           |            |                |                | Tracheal tissue     | N/A                          | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 4.  | II04      | 18         | Free-range     | 66.7%          | Oro-pharyngeal swab | N/A                          | +                             |
|          |            |     |           |            |                |                | Tracheal tissue     | +                            | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | N/A                           |
|          |            | 5.  | II05      | 52         | Free-range     | 100.0%         | Oro-pharyngeal swab | -                            | +                             |
|          |            |     |           |            |                |                | Tracheal tissue     | +                            | +                             |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | N/A                           |
|          |            | 6.  | II06      | 16         | Free-range     | 75.0%          | Oro-pharyngeal swab | +                            | +                             |
|          |            |     |           |            |                |                | Tracheal tissue     | N/A                          | +                             |
|          |            |     |           |            |                |                | Cloacal swab        | N/A                          | +                             |
|          |            | 7.  | II07      | 19         | Semi-intensive | 78.9%          | Oro-pharyngeal swab | N/A                          | +                             |
|          |            |     |           |            |                |                | Tracheal tissue     | +                            | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 8.  | II08      | 12         | Semi-intensive | 83.3%          | Oro-pharyngeal swab | +                            | -                             |
|          |            |     |           |            |                |                | Tracheal tissue     | N/A                          | +                             |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 9.  | II09      | 22         | Free-range     | 100.0%         | Oro-pharyngeal swab | +                            | N/A                           |
|          |            |     |           |            |                |                | Tracheal tissue     | N/A                          | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 10. | II10      | 29         | Free-range     | 100.0%         | Oro-pharyngeal swab | +                            | N/A                           |
|          |            |     |           |            |                |                | Tracheal tissue     | +                            | +                             |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 11. | II11      | 16         | Free-range     | 70.0%          | Oro-pharyngeal swab | +                            | N/A                           |
|          |            |     |           |            |                |                | Tracheal tissue     | N/A                          | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | +                            | +                             |
|          |            | 12. | II12      | 11         | Free-range     | 90.9%          | Oro-pharyngeal swab | +                            | N/A                           |
|          |            |     |           |            |                |                | Tracheal tissue     | +                            | N/A                           |
|          |            |     |           |            |                |                | Cloacal swab        | N/A                          | +                             |

|          |     |      |    |                |        |                     |     |     |
|----------|-----|------|----|----------------|--------|---------------------|-----|-----|
| Bulamagi | 13. | II13 | 28 | Free-range     | 92.9%  | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |        | Tracheal tissue     | N/A | N/A |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 14. | II14 | 62 | Free-range     | 83.9%  | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |        | Tracheal tissue     | +   | N/A |
|          |     |      |    |                |        | Cloacal swab        | +   | N/A |
|          | 15. | II15 | 19 | Free-range     | 84.2%  | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |        | Tracheal tissue     | N/A | N/A |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 16. | II16 | 32 | Free-range     | 90.6%  | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |        | Tracheal tissue     | N/A | N/A |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 17. | IB01 | 9  | Free-range     | 100.0% | Oro-pharyngeal swab | +   | -   |
|          |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | N/A | +   |
|          | 18. | IB02 | 18 | Free-range     | 72.2%  | Oro-pharyngeal swab | N/A | +   |
| Makuutu  |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 19. | IB03 | 28 | Free-range     | 78.6%  | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 20. | IB04 | 11 | Free-range     | 90.9%  | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | N/A | +   |
|          | 21. | IB05 | 58 | Semi-intensive | 77.6%  | Oro-pharyngeal swab | N/A | +   |
|          |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | -   | +   |
|          | 22. | IB06 | 13 | Free-range     | 61.5%  | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 23. | IB07 | 18 | Free-range     | 100.0% | Oro-pharyngeal swab | N/A | N/A |
|          |     |      |    |                |        | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |        | Cloacal swab        | -   | +   |
|          | 24. | IB08 | 22 | Free-range     | 54.5%  | Oro-pharyngeal swab | N/A | N/A |
|          |     |      |    |                |        | Tracheal tissue     | +   | +   |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 25. | IB09 | 18 | Free-range     | 66.7%  | Oro-pharyngeal swab | N/A | N/A |
|          |     |      |    |                |        | Tracheal tissue     | +   | +   |
|          |     |      |    |                |        | Cloacal swab        | +   | +   |
|          | 26. | IM01 | 24 | Free-range     | 91.7%  | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |        | Tracheal tissue     | +   | N/A |

|          |     |      |    |                |       |                     |     |     |
|----------|-----|------|----|----------------|-------|---------------------|-----|-----|
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          |     |      |    |                |       | Oro-pharyngeal swab | N/A | N/A |
|          | 27. | IM02 | 31 | Free-range     | 96.8% | Tracheal tissue     | +   | N/A |
|          |     |      |    |                |       | Cloacal swab        | +   | N/A |
|          | 28. | IM03 | 49 | Free-range     | 73.7% | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |       | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          | 29. | IM04 | 12 | Free-range     | 91.7% | Oro-pharyngeal swab | -   | +   |
|          |     |      |    |                |       | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          |     |      |    |                |       | Oro-pharyngeal swab | +   | +   |
| Tororo   | 30. | TM01 | 69 | Semi-intensive | 91.3% | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
| Mukujju  | 31. | TM02 | 12 | Semi-intensive | 83.3% | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          | 32. | TM03 | 62 | Free-range     | 88.7% | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          | 33. | TM04 | 41 | Free-range     | 90.9% | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          | 34. | TM05 | 18 | Free-range     | 66.7% | Oro-pharyngeal swab | +   | N/A |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | N/A |
|          | 35. | TM06 | 12 | Free-range     | 75.0% | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |       | Tracheal tissue     | N/A | +   |
|          |     |      |    |                |       | Cloacal swab        | -   | +   |
|          | 36. | TM07 | 15 | Free-range     | 86.7% | Oro-pharyngeal swab | N/A | +   |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
| Osukurru | 37. | TO01 | 62 | Free-range     | 67.7% | Oro-pharyngeal swab | N/A | +   |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          | 38. | TO02 | 71 | Semi-intensive | 87.3% | Oro-pharyngeal swab | +   | +   |
|          |     |      |    |                |       | Tracheal tissue     | +   | +   |
|          |     |      |    |                |       | Cloacal swab        | +   | +   |
|          |     |      |    |                |       | Oro-pharyngeal swab | +   | +   |

N/A = Not applicable (No sample analyzed); = Negative result (Sample RNA not amplified); + = Positive result (Sample RNA amplified)

## Appendix 11



Map of Uganda: Tororo and Iganga District inset where samples were collected (Map adopted from Uganda Bureau of Statistics, 2007)