ADDIS ABABA UNIVERSITY
FACULTY OF VETERINARY MEDICINE

ISOLATION, IDENTIFICATION AND SEROPREVALENCE OF NEWCASTLE DISEASE VIRUS IN VILLAGE CHICKENS IN SOUTH WEST SHEWA, ETHIOPIA

BY

BELAYNEH GETACHEW AYALEW

JUNE, 2009
DEBREZEIT, ETHIOPIA

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LIST OF ABBREVIATIONS

Abs	Antibodies
AH	Allantoic Harvest
APMV	Avian paramyxoviridae viruses
dNTP	Di nucleotide triphosphate
ELISA	Enzyme Linked Immunosorbent Assay
F	Fusion protein
HA	Haemagglutination
HI	Haemagglutination Inhibition
HN	Haemagglutinine/Neuraminidase
HPAI	Highly Pathogenic Avian Influenza
ICPI	Intra Cerebral Pathogenecity Index
MDT	Mean death time
ND	Newcastle Disease
NDV	Newcastle Disease Virus
RT-PCR	Reverse transcriptase polymerase chain reaction
SPF	Specific pathogen free
VND	Velogenic Newcastle Disease
VVND	Viscerotropic Velogenic Newcastle Disease

ABSTRACT

A study on seroprevalence, isolation and identification of Newcastle disease virus in village chickens in south west shewa zone was conducted using Enzyme Linked Immunosorbent Assay, Haemagglutination, Haemagglutination inhibition test and real time RT-PCR. A total of 355 chickens from eight kebeles of the study site were used for the study of seroprevalence. The overall seroprevalence of Newcastle disease virus antibodies in the study area was 5.6% (3.2-8.0% at 95 % CI). Five (62.5%) of the eight kebeles sampled had chickens that were positive for antibodies against NDV. The prevalence in each kebele ranges from 0% to 28.1% and the highest prevalence (28.1%) was found at Harbu Kebele which is located just near to the market. The prevalences of chicken's serum antibody in the highland and lowland area were 0.9% and 7.8% respectively. Statistically significant ($p<0.05$) difference in prevalence of Newcastle disease virus antibodies was found between highland and lowland, local and cross breeds and young and adult chickens. The difference, however, was not statistically significant ($p>0.05$) for male and female. Much (95%) of the chicken sera do have a percent inhibition value between -104 and 74.33 and a normally distributed figure was found. From Newcastle suspected outbreak cases, six samples were collected from recently dead and sick chickens for isolation and identification of Newcastle disease virus. The result indicated that all the six samples were positive for Haemagglutination and Haemagglutination inhibition tests. But cloacal and tracheal swab samples from 30 apparently healthy chickens revealed that there was no haemagglutinating viral agents. None of the samples can lead to the death of embryo even at the second passage. Subjection of genome extract from allantoic harvest to real time RT-PCR using specific primer for fusion protein cleavage site resulted in amplification of viral genome. Three samples taken from allantoic harvest of outbreak areas were amplified but none was amplified from apparently healthy chicken using real time RT-PCR. This further confirmed that Avian Paramyxovirus type one was isolated and identified from outbreak cases of ND in the study area. The low prevalence to NDV in the study area indicated that the village chickens are highly susceptible to the pathogenic NDV infection. Thus, it is recommended that there should be routine vaccination program in the study area.

Key word: *Identification, Isolation, Newcastle disease, Seroprevalence, Village Chicken*

1. INTRODUCTION

Just like in other developing countries the poultry industry in Ethiopia is dominated by the traditional sector. Free-range poultry keeping is most common in the country. The chickens realed under traditional or "backyard" conditions accounts for 99%, while only 1% are birds kept under intensive management system in commercial farms (Alamargot, 1987; CSA, 2008). Large portions of the human world population today are insufficiently supplied with quality protein in the diet. This is particularly true for a large portion of the population in Ethiopia. Poultry occupies a unique position through its contribution to the supply of valuable food protein as well as by providing income to the families in developing countries. As far as the Ethiopian poultry farming system is concerned, it is apparent that village chickens are more important than those kept under intensive management system with regard to total numbers, egg and poultry meat production (Alemu, 1995). They provide livelihood and supply 100% of eggs and chicken meat consumed in rural areas, where 85% of the population live. Therefore, free-range chickens have an important role in economic and nutritional needs of the Ethiopian people especially in the rural areas.

The contribution of the chicken industry to the national economy and the per-capita meat and egg consumption is very low. At the same time poverty and protein deficiency is manifested by wide spread malnutrition in children and women in village communities. Several factors have been suggested for the low production characteristics of free-range village chickens. The system is characterized by low input and low output, with minimal management interventions, feed supplementation, housing and disease control. This kind of production by itself is a limiting factor to sound economic and sustainable production. The low input might be, however, a result of the high risk due to high mortalities experienced in village poultry. Diseases and especially the devastating Newcastle disease (ND) are perceived to be the main constraint (Tadesse *et al.,* 2005; Dessie, 1996; Nasir, 1998), which frustrates any investment in this system. Thus, the potential of the free-range chicken production has not been exploited. Therefore, if any success is to be achieved in improvements for free-range village chickens production, it will inevitably depend on the successful control of major poultry diseases in general and ND in particular ND.

Newcastle disease is a viral disease affecting mainly chickens. It is caused by avian paramyxovirus serotype 1 (APMV-1) (NDV). The disease is mainly controlled by vaccination. ND has long been known to be endemic in Ethiopia (Nassir, 1998; Zeleke *et al.,* 2005; Tadesse *et al.,* 2005). Its history, origin and spread to Ethiopia have not been well explained. However, it is wide spread in the country and it has different local names in different areas and the most common one is "Fengil" (Edward, 1992; Dessie, 1996; Nasir, 1998), which means sudden dorsal prostration that signifies the acuteness and severity of the disease.

Newcastle disease (ND) - a List A infection is considered as one of the two most important diseases of chickens along with highly pathogenic avian influenza (Aldous and Alexander, 2001). It is an economically important disease causing heavy production loss to the farmers besides high mortality. The disease is present in endemic form with frequent outbreaks in different parts of the country and it remains as a constant threat to the backyard poultry (Spadbrow, 1993; Westbury, 2001). While the commercial poultry are routinely vaccinated, the village chickens are not normally vaccinated due to social and financial reasons (Spadbrow, 1992; Alexander, 2001).

The epidemiology and control of ND has been extensively studied and documented in commercial poultry systems, but has been poorly documented in village poultry. The large differences in management between commercial and village poultry prohibit the transfer of epidemiological data and control programmes of ND from the commercial sector to the village environment. Thus, successes in attempts to control ND in village poultry have been hardly successful (Alexander, 1997). It is well known that knowledge of specific disease risk factors is a prerequisite for effective evaluation of disease control programmes.

For this to be feasible, the type of virus circulating in the area should be known and a base line date should be established to assist in formulating ND control programmes. Consequently, epidemiological cross-sectional studies on free-range village poultry have to be carried out in Ethiopia to generate data, which could be used in the formulation of Newcastle disease control program. Nevertheless, there has been limited information available on type of viruses responsible for different outbreak cases of suspected Newcastle disease and prevalence of ND in different parts of Ethiopia.

Therefore the objectives of this paper are:

- To determine prevalence of ND virus antibodies in village chickens and identify possible risk factors in the study area
- To isolate and identify the type of the virus responsible for outbreak cases of suspected Newcastle disease in the study area

2. LITRATURE REVIEW

2.1. Definition of the disease

Newcastle disease is a serious and commonly fatal viral poultry disease, which is present all over the world. In many tropical and subtropical countries virulent strains of Newcastle disease virus are endemic (Spradbrow, 1990). In most developing countries, ND is the most important infectious disease affecting village chickens and it causes great economic losses (Spradbrow, 1999; Aini, 1990). The disease is one most contagious of all viral disease, spreading rapidly among susceptible chickens, in some cases seemingly in mysterious fashion (Murphy *et al.*, 1999). It can also be defined as an infection of birds caused by a virus of avian paramyxovirus serotype 1 (APMV-1) that meets one of the following criteria for virulence:

a) The virus has an intracerebral pathogenicity index (ICPI) in day-old chicks (Gallus gallus) of 0.7 or greater.

Or

b) Multiple basic amino acids have been demonstrated in the virus (either directly or by deduction) at the C-terminus of the F2 protein and phenylalanine at residue 117, which is the N-terminus of the F1 protein. The term 'multiple basic amino acids' refers to at least three arginine or lysine residues between residues 113 and 116. Failure to demonstrate the characteristic pattern of amino acid residues as described above would require characterization of the isolated virus by an ICPI test.

In this definition, amino acid residues are numbered from the N-terminus of the amino acid sequence deduced from the nucleotide sequence of the F0 gene, 113–116 corresponds to residues –4 to –1 from the cleavage site (OIE, 2008).

2.2. Etiology

2.2.1. Classification

Newcastle disease (ND) is caused by specified viruses of the avian paramyxovirus type I (APMV-I) serotype of the genus *Avulavirus* belonging to the subfamily Paramyxovirinae, family Paramyxoviridae. The paramyxoviruses isolated from avian species have been classified by serological testing into nine serotypes designated APMV-1 to APMV-9; ND virus has been designated APMV-1 (Alexander, 1997).

One of the most characteristic properties of different strains of ND virus has been their great variation in pathogenicity for chickens. Strains of ND virus have been grouped into five pathotypes on the basis of the clinical signs seen in infected chickens (Beard and Hanson, 1981).These are:

Viscerotropic velogenic: a highly pathogenic form in which haemorrhagic intestinal lesions are frequently seen;

Neurotropic velogenic: a form that presents with high mortality, usually following respiratory and nervous signs;

Mesogenic: a form that presents with respiratory signs, occasional nervous signs, but low mortality;

Lentogenic or respiratory: a form that presents with mild or subclinical respiratory infection;

Asymptomatic enteric: a form that usually consists of a subclinical enteric infection.

Pathotype groupings are rarely clear-cut (Alexander and Allan, 1974) and even in infections of specific pathogen free (SPF) birds, considerable overlapping may be seen. In addition, exacerbation of the clinical signs induced by the milder strains may occur when infections by other organisms are superimposed or when adverse environmental conditions are present.

2.2.2. Virus structure and replication

The paramyxoviridae family contain a lipid bilayer envelop that is derived from the plasma membrane of the host cell in which the virus is grown. A lipid envelop contains two surface glycoproteins (Fusion and Hemagglutinin-Neuraminidase), which mediate the entry and exit of the virus from its host cell. Inside the envelope lies a helical nucleocapsid(N), phospho-(P), and large (L) proteins, which initiate intracellular viral replication. They contain non segmented, single stranded genome of negative polarity (Murphy *et al.*, 1999). Paramyxovirus virions are generally spherical and 150 nm to 330nm in diameter, but they can be pleomorphic, and filamentous forms can also be observed (Alexander, 1997).

Replication of the virus takes place in the cytoplasm of the host cell. Virions attach via their hamagglutinin- neuraminidase protein to cellular sialoglycoprotein or glycolipid receptors. The fusion protein then mediates fusion of the viral envelop with the plasma membrane, at physiologic pH. The librated nucleocapsid remains intact, with all three of its associated proteins (N, P, and L) being required for transcription by the virion-associated, RNA-dependant RNA polymerase. The genome is transcribed progressively in to six discrete unprocessed mRNAs by sequential interrupted synthesis from a single promoter. Full genome-length positive-sense RNA is also synthesized and serves a template for the replication of the negative sense genomic RNA (Murphy *et al.*, 1999).

2.3. Epidemiology

2.3.1. Host Range

Newcastle disease viruses have been reported to infect animals other than birds, ranging from repitiles to man (Lancaster, 1966). Kaleta and Baldauf (1988) concluded that NDV infections have been established in at least 241 species of birds representing 27 of the 50 Orders of the class. It seems probable that all birds are susceptible to infection but,as stressed by Kalata and Baldauf, the disease seen with any given virus may vary enormously from one species to another. A persistent carrier state has been demonstrated in psittacine and in certain other wild birds (Vickers and Hanson, 1979) whereas virus can be recovered from chickens for shorter periods of time, usually 14 days or less. Inapparently infected carriers that are the most likely source for introduction of velogenic newcastle disease include numerous species of exotic pet and exposition birds, waterfowl, and domestic poultry (Uttrback and Schartz, 1973).

2.3.2. Transmission

The mode of transmission from bird to bird is clearly dependant on the origins in which the virus multiplies. Birds showing respiratory disease presumably shed virus in aerosol of mucus which may be inhaled by susceptible birds. Viruses that are mainly restricted to intestinal replication may be transferred by ingestion of contaminated faeces, either directly or in contaminated food or water, by inhalation of small infective particles produced from dried faeces. Such considerations may drastically affect the rate of spread. Viruses transmitted by the respiratory route in a community of closely situated birds (i.e. an intensive broiler house) may spread with alarming rapidity. Viruses excreted in the faeces and transmitted chiefly by the oral /faecal rout may spread extremely slowly, especially if birds are not on the direct contact (i.e. caged layers) (Jordan *et al.*, 2001).

Infected birds shed virus in exhaled air, respiratory discharges, and feces. Virus is shed during incubation, during the clinical stage, and for a varying but limited period during convalescence. Virus may also be present in eggs laid during clinical disease and in all parts of the carcass during acute virulent infections. Infected chickens are the primary source of virus, but other domestic and wild birds may be sources of NDV (Alexander, 1997).

Assessments of airborne spread of NDV over large distances have produced varied results. During the panzootic of 1970-1973 this type of spread was considered to be major importance in some countries but of much less significance in others. If airborne spread occurs at all over more than quite short distances it probably requires very specific weather conditions. Humans seem to play the central role in the spread of NDV, usually by the movement of live birds, fomites, personal and poultry products (including dead birds and faeces for fertilizer) from affected premises to susceptible birds. Feral birds and other wild life have undoubtedly contributed to the spread of disease during epizootic, either by infection or by mechanical transfer; their exact role has not been fully evaluated (Jordan *et al.,* 2001).

A carefully documented epizootic in fowl occurring in the UK in 1984 demonstrates the interaction between the methods of spread that may occur. A variant form of NDV, which could be differentiated from more classical viruses by monoclonal antibodies, was introduced in to the country probably by stray racing pigeons. The virus proceeded to spread rapidly amongst UK racing pigeon from these to feral pigeons. This resulted in the infection of a flock of pigeons living on food stored at a dockyard. Food contaminated with pegeon feaces from these stores was fed untreated to fowls that subsequently developed disease. There was relatively little secondary spread but where it was inevitably related to the agency of humans in the movement of personnel, contaminated vehicles and unfumigated eggs (Jordan *et al.,* 2001).

A key to the successful spread of NDV is the ability of the virus to survive in the dead host or excretions. In infected carcass NDV may survive for several weeks at cool ambient temperatures or several years if held frozen. Feaces in which virus may be present in high titters, also represents an excellent medium for the survival of NDV, and even at 37^{o}c infectivity has been retained for over a month.

In many parts of the tropics VND is recurrent in the poultry populations. One possibility is that they are infected from a wild bird reservoir. Additional studies will be required before it can be established which species, if any, are true carriers and which are only transiently infected. Once introduced into poultry, the virus spreads farm-to-farm by the movement of inapparently infected poultry species; on contaminated objects such as boots, sacks, egg trays, and crates; or by flies (Bram *et al.,* 1974) or mice.

2.3.3. Geographic Distribution

Virulent NDV strains are endemic in poultry in most of Asia, Africa, and some countries of North, Central, and South America. Other countries, including the USA and Canada, are free of those strains and maintain that status with import restrictions and eradication by destroying diseased poultry (Alexander, 1997).

The history of Newcastle disease is marked by at least three panzootics in domestic birds. The first began with the emergence of the disease in fowl in the mid- 1920s and spread slowly from the Far East through out the world, with extremely rapid spread within some countries. The second panzootic appeared to emerge in fowl in the Middle East in the late 1960s and spread much faster than the first, reaching all continents and most countries by 1973. Several authors associated this rapid spread with the movement of infected psittacine birds as a consequence of the international pet bird market. A third panzootic was associated with a mainly neurotropic and enteric disease in pigeons. It too appeared to emerge in the Middle East and between the late 1970s and the mid -1980s spread through out the world in racing, show, meat and feral pigeon; in some countries speed to other birds and poultry occurred (Jordan *et al.,* 2001).

2.4. Pathogenesis, Pathology and Immunity

Initially the virus replicates in the mucosal epithelium of the upper respiratory and intestinal tracts; shortly after infection, virus spreed via the blood to the speen and bone marrow, producing the secondary viremia. This leads to infection of other target organs: lung, intestine, and centeral nervous system. Resopiratory distress and dyspenea result from congestion of the lungs and damage of the respiratory center in the brain. Gross pathologic findings include ecchymotic hemorrhage in the larynx, trachea, esophagus, and through out the intestine. The most prominent histologic lesions are necrotic foci in the intestinal mucosa and the lymphatic tissue and hypermic changes in most organs including brain(Murphy *et al.,* 1999).

Strains of Newcastle disease virus differ widely in virulence, depending on the cleavability and activation of their haemagglutinin-neuraminidase and fusion glyco- proteins. Avirulent strains produce inactive precursor protiens; in virulent strains these precursors are cleaved and activated readily. Cleavage activation must occur in the same tissue in which intial viral replicattioin occurs if the infection is to be progressive-the presence of cellular protease as well as the strain based cleavablity of the viral glycoprotein precursors defines the tissue tropism of various viral strains and their capacity to spread rapidly.

The terms velogenic (viscerotropic), mesognic, annd lentogenic are applied to Newcastle disease virus strains of high, intermidiate, and low virulence. Whereas velogenic strains kill virtually 100% of infected chickens, naturally avirulent strains have been used as vaccines. Virulent velogenic strains cause predominantly hemorrhagic lesions, in particular at the esophagus/gizard junctions and in the posterior half of the duodenum, the jejunum and ileum. These lesions are virtually pathognomic for velogenic strains. In severe cases, hemorrhages are also present in subcutanous tissue, muscles, larynx, trachea/esophageal tissue, serous membranes, lungs, airsacs, pericardium and myocardium. In adult hens, hemorrhages are present in ovarian follicles. Lessions can develop in to diphtheroid inflammatory foci and later in to necrotic foci. In the centeral nervous system, lessions are those of encephalomyelitis-neuronal necrosis, prevascular cuffing, and interstitial inflamatory infiltration (Murphy *et al.,* 1999).

Antibody production is rapid. Hemagglutination inhibiting antibody can be detected within 4 to 6 days of infection and persists for at least 2 years. The level of hemagglutinating-inhibiting antibody is a measure of immunity. Maternal antibodies protect chicks for three to four weeks after hatching. IgG is confined to the circulaion and does not prevent respiratory infection, but it does block viremia; locally produced IgA antibodies play an important role in protection in both the respiratory tract and the intestine(Murphy *et al*., 1999).

2.4.1. Molecular basis for pathogenicity

During replication, NDV particles are produced with a precursor glycoprotein, F0, which has to be cleaved to F1 and F2 for the virus particles to be infectious. This post-translation cleavage is mediated by host-cell proteases. Trypsin is capable of cleaving F0 for all NDV strains.

It would appear that the F0 molecules of viruses virulent for chickens can be cleaved by a host protease or proteases found in a wide range of cells and tissues, and thus spread throughout the host damaging vital organs, but F0 molecules in viruses of low virulence are restricted in their cleavability to certain host proteases resulting in restriction of these viruses to growth only in certain host-cell types.

Most ND viruses that are pathogenic for chickens have the sequence ^{112}R/K-R-Q-K/R-R^{116} at the C terminus of the F2 protein and F (phenylalanine) at residue 117, the N-terminus of the F1 protein, whereas the viruses of low virulence have sequences in the same region of ^{112}G/E-K/R-Q-G/E-R^{116} and L (leucine) at residue 117. Some of the pigeon variant viruses (PPMV-1) examined have the sequence ^{112}G-R-Q-K-RF^{117}, but give high ICPI values. Thus, there appears to be the requirement of at least one pair of basic amino acids at residues 116 and 115 plus a phenylalanine at residue 117 and a basic amino acid (R) at 113 if the virus is to show virulence for chickens.

Several studies have been done using molecular techniques to determine the F0 cleavage site sequence by reverse-transcription polymerase chain reaction (RT-PCR), either on the isolated virus or on tissues and faeces from infected birds, followed by analysis of the product by restriction enzyme analysis, probe hybridisation or nucleotide sequencing with a view to establishing a routine *in vitro* test for virulence. Determination of the F0 cleavage sequence may give a clear indication of the virulence of the virus, and this has been incorporated into the definition of ND by OIE, which is mentioned under the definition of the disease.

In the diagnosis of ND it is important to understand that the demonstration of the presence of virus with multiple basic amino acids at the F0 cleavage site confirms the presence of virulent or potentially virulent virus, but that failure to detect virus or detection of NDV without multiple basic amino acids at the F0 cleavage site using molecular techniques does not confirm the absence of virulent virus. Primer mismatch or the possibility of a mixed population of virulent and avirulent viruses means that virus isolation and an *in vivo* assessment of virulence will still be required.

Analyses of viruses isolated in Ireland in 1990 and during the outbreaks of ND in Australia since 1998 have given strong evidence that virulent viruses may arise from progenitor viruses of low virulence (Alexander, 2001; Westbury, 2001). Virulent NDV has also been generated experimentally from low virulence virus by passage in chickens (Shenpqing *et al.*, 2002).

2.5. Clinical Findings

Onset is rapid, and signs appear throughout the flock within 2-12 days (average 5) after aerosol exposure. Spread is slower if the fecal-oral route is the primary means of transmission, particularly for caged birds. Young birds are the most susceptible. Observed signs depend on whether the infecting virus has a predilection for respiratory, digestive, or nervous systems. Respiratory signs of gasping, coughing, sneezing, and rales predominate in low virulence infections. Nervous signs of tremors, paralyzed wings and legs, twisted necks, circling, clonic spasms, and complete paralysis may accompany, but usually follow the respiratory signs in neurotropic velogenic disease. Nervous signs with diarrhea are typical in pigeons, and nervous signs are frequently seen in cormorants and exotic bird species. Respiratory signs with depression, watery-greenish diarrhea, and swelling of the tissues of the head and neck are typical of the most virulent form of the disease. Varying degrees of depression and inappetence are observed. A partial or complete cessation of egg production may occur. Eggs may be abnormal in color, shape, or surface, and have watery albumen. Mortality is variable but can be as high as 100% (Alexander, 1997).

2.6. Diagnosis

A tentative diagnosis of VND may be made on the basis of history, clinical signs, and gross lesions, but because of similarities to other diseases such as fowl cholera and highly pathogenic avian influenza, confirmation requires virus isolation and identification. Isolation of a hemagglutinating virus identified by inhibition with Newcastle disease antiserum confirms the diagnosis. A rise in hemagglutination-inhibition antibodies in paired serum samples also confirms the disease. Virulence of an isolate is established by the rapidity of killing day-old chicks inoculated in to the intracerebral route (the intracerebral pathogenicity index), by inoculated in to allantic cavity of 9 -10 day-old embryonated SPF eggs (Mean Death Time), and injecting in to intravenously into each of ten 6-week –old SPF chickens (intravenous pathogenecity index) or by the presence of a specified amino acid motif at the cleavage site of the fusion protein (F) precursor (F0). Reference laboratories use monoclonal antibodies to detect antigenic differences and nucleotide sequence analysis to detect genetic differences for comparison of isolates from different outbreaks and to identify the source of those infections (Alexander, 1997).

2.6.1. Field Diagnosis

Specimens for Laboratory: Virus can readily be recovered from sick or recently dead birds. Swabs are the most convenient way to transfer VND virus from tissues or secretions of the suspect bird to virus transport medium containing high levels of antibiotics (Alexander, 1989). Trachea, lung, spleen, cloaca, and brain should be sampled. Swabs should be inserted deeply to ensure obtaining ample epithelial tissue. If large numbers of dead or live birds are to be sampled, cloacal swabs from up to five birds can be pooled in the same tube of viral transport medium. An alternate technique is to place 0.5 cm^3 of each tissue into the viral transport medium. If the specimens can be delivered to a laboratory within 24 hours, they should be placed on ice. If delivery will take longer, quick-freeze the specimens and do not allow them to thaw during transit.

2.6.2. Laboratory Diagnosis

In the laboratory, virus isolation is attempted by inoculating 9- to 11-day-old embryonating chicken eggs. Chorioallantoic fluid (CAF) is collected from all embryos dying after 24 hours post inoculation and tested for hemagglutination (HA) activity. If positive, the hemagglutination-inhibition (Hl) test is used with known NDV-positive serum to confirm the presence of NDV in the CAF (Beard, 1989). If NDV is found, it is characterized by inoculating 4- to 6-week-old chickens free of ND antibodies with the suspect CAF by swabbing the cloaca, instilling into the nares or conjuctival sac, or injecting into the thoracic air sac. If VVND virus is present, the inoculated chicks usually die in 3 to 7 days, revealing typical visceral lesions on postmortem examination. Neurotrophic VVD viruses will cause severe neurologic and respiratory signs in inoculated chickens but no visceral lesions. If no bird dies in 10 days, the NDV is not considered to be the velogenic, viscerotropic type but is either a lentogen or mesogen.

NDV may be employed as an antigen in a wide range of serological tests, enabling neutralisation or enzymelinked immunosorbent assays (ELISA) and HI to be used for assessing antibody levels in birds. At present, the HI test is most widely used for detecting antibodies to NDV in birds, although many poultry producers are using commercial ELISA kits to assess post-vaccination antibody levels (OIE, 2008).

There are varieties of commercial ELISA kits available and these are based on several different strategies for the detection of NDV antibodies, including indirect, sandwich and blocking or competitive ELISAs using MAbs. At least one kit uses a subunit antigen. Usually such tests have been evaluated and validated by the manufacturer, and it is therefore important that the instructions specified for their use be followed carefully. The HI test and ELISA may measure antibodies to different antigens; depending on the system used. ELISAs may detect antibodies to more than one antigen while the HI test is probably restricted to those directed against the HN protein. However, comparative studies have demonstrated that the ELISAs are reproducible and have high sensitivity and specificity; they have been found to correlate well with the HI test (Adair, 1989).

Conventional ELISAs have the disadvantage that it is necessary to validate the test for each species of bird for which they are used. Competitive ELISAs usually employ MAbs, which, because of their specificity for single epitopes, may not recognize all strains of APMV-1 (OIE, 2008).

In addition to the use of RT-PCR and other similar techniques for the determination of the virulence of ND viruses or for phylogenetic studies, there has been increasing use of such molecular techniques to detect NDV in clinical specimens, the advantage being the extremely rapid demonstration of the presence of virus (OIE, 2008).

Care should be taken in the selection of clinical samples as some studies have demonstrated lack of sensitivity in detecting virus in some organs and particularly in faeces (Koch, 2003; Creelan *et al.,* 2002; Gohm *et al.,* 2000). Tracheal or oropharyngeal swabs are often used as the specimens of choice because they are easy to process and usually contain little extraneous organic material that can interfere with RNA recovery and amplification by PCR. However, tissue and organ samples and even faeces have been used with some success. The system used for RNA extraction will also affect the success of RT-PCR on clinical specimens and even with commercial kits care should be taken in selecting the most appropriate or validated for the samples to be analysed.

Usually RT-PCR systems have been used to amplify a specific portion of the genome that will give added value; for example by amplifying part of the F gene that contains the F0 cleavage site so that the product can be used for assessing virulence (Creelan *et al.*, 2002; Gohm *et al.*, 2000). Perhaps the most serious problem with the use of RT-PCR in diagnosis is the necessity for post-amplification processing because of the high potential for contamination of the laboratory and cross contamination of samples. Extreme precautions and strict regimens for handling samples are necessary to prevent this.

One of the strategies used to avoid post-amplification processing is to employ real-time RT-PCR (rRT-PCR) techniques. The advantages of such assays are that rRT-PCR assays based on the fluorogenic hydrolysis probes or fluorescent dyes eliminate the post-amplification processing step and that results can be obtained in less than 3 hours. The most successful application of an rRT-PCR assay was in the USA during the ND outbreaks of 2002–2003, when the assay described by Wise *et al.*, (2004) was employed and showed a sensitivity of 95% when compared with virus isolation for more than 1400 specimens. The assay has three sets of primers and probes that are used in separate reactions: a matrix primer/probe set that is designed to detect most strains of NDV, a fusion primer/probe set that can identify virulent strains of NDV (including many PPMV-1 viruses) and a primer/probe set designed to detect low virulent strains of the virus. Samples are first screened with the matrix primers/probe then positive specimens are tested with the low virulent and fusion and primers/probe sets to confirm presence of low or highly virulent virus, respectively. The primers and probes in this report were validated on lentogenic, mesogenic and velogenic strains circulating in the United States of America. At the peak of the outbreak, between 1000 and 1500 samples were tested daily by rRT-PCR. A disadvantage of rRT-PCR is that, at present, the special thermocyclers required are extremely expensive and this would deter many laboratories from employing this system.

2.6.4. Differential Diagnosis

The viscerotropic, velogenic Newcastle disease in poultry can be confused with highly pathogenic avian influenza, infectious laryngotracheitis, fowl cholera, and coryza.

2.7. Prevention and control

Because Newcastle disease is a notifiable disease in most developing countries, legislative measures constitute the basis for control. Where the disease is endemic, control is achieved by good hygiene combined with immunization.

2.7.1. Vaccination

Vaccination, initially with inactivated virus, was considered a possibility for the control of ND at the time of the apparent emergence of the virus. However, after the 1933 outbreak in England, an attenuated live vaccine was produced which was called strain H. Later, the naturally occurring USA isolates of low virulence, Hitchner B1 (HB1) and La Sota, became the most used veterinary vaccines throughout the world. Fifty years or more have passed since vaccine was first used to protect village poultry against ND (Placcidi and Sentucci, 1952). During this time, a wide variety of types of vaçcine have been developed. Many, but not all, have been tested on village poultry.

The principle of vaccination against a viral disease is well-known: to elicit an immunological response against the virus in a way that does not cause the disease. The simplest way to do this is to take the virus, kill it, and then inject it into the bird. This is an inactivated vaccine. Another approach is to select a naturally occurring virus that is not virulent enough to cause serious disease, and infect the birds with this virus. This is a live vaccine. This latter approach can be taken further by taking a non virulent natural virus and selecting a clone from the virus population with desirable properties, such as lack of vaccinal reactions, or heat tolerance. This is a cloned live vaccine. Finally, it is possible to genetically engineer a vaccine by, for example, taking part of the genetic material of the virus that codes for a surface antigen, and inserting this into another, different, virus to produce a recombinant vaccine.

These different approaches to vaccination have been applied to ND. There are three types of vaccines used for ND: live lentogenic, live mesogenic and inactivated vaccines. Live lentogenic vaccines are usually derived from field viruses that have been shown to have low pathogenicity for poultry but produce an adequate immune response. Typical vaccine strains are HB1, La Sota and F strain and some viruses from the asymptomatic enteric pathotype, which are usually based on the V4 or Ulster 2C viruses. However, these viruses have been frequently subjected to selection pressures by manufacturers in order to improve their immunogenicity or to enable their use by a particular method of application

Inactivated vaccines: Inactivated vaccines are produced by growing a ND virus in eggs, and then treating the infective allantoic fluid with an inactivating agent, such as formalin or betapropiolactone. An adjuvant, such as mineral oil, is usually then added to make the inactivated virus more immunogenic. Since the vaccine is no longer capable of replication or spread, it has to be injected individually into every bird needing vaccination. It is normally injected into the back of the thigh muscle (sometimes the breast muscle is used), using 0.3 or 0.5 ml per bird. This requires some training, and can not be done by every keeper of chickens without prior demonstration. Inactivated vaccines produce very high levels of antibodies against NDV, and provide good protection against the virulent virus.

In intensive poultry production, inactivated vaccines are usually applied after an initial priming vaccination with a live vaccine. In village poultry, however, good results in the absence of an initial vaccination with live vaccine have been reported (Bell *et al.*, 1990). The reason for this is probably, as serological surveys have shown where they have been carried out (Bell and Mouloudi, 1988), that antibodies to the virus are already present in the village poultry as a result of previous infection by the wild virus.

Inactivated vaccines have been used extensively in village poultry, for example, in a successful project in Burkino Faso (Verger, 1986). Although inactivated vaccine gives good protection, it is relatively expensive to produce. It also carries a slight risk to the user of accidental self-injection. While inactivated vaccines are, to some extent, heat sensitive, they are much less so than conventional live vaccines which makes transporting them to villages more feasible.

Live vaccines: Live vaccines differ from inactivated vaccines in that they can replicate in the host. This is both an advantage and a disadvantage. It is an advantage in that it is not necessary to vaccinate every bird individually; the vaccinal virus can spread on its own from one bird to another. It is, however, a disadvantage in that, since an infection with a live virus is involved; this may result in clinical signs because of the innate virulence of the vaccine virus or by exacerbating other organisms that may be present, especially in the respiratory tract. The severity of this reaction depends therefore on the particular vaccinal strain used (Westbury *et al.*, 1984) and the presence or otherwise of concurrent infection with other pathogens.

Another advantage of live vaccines compared to inactivated vaccines, is their ease of application as they can be applied to the drinking water or with an eye-dropper.

Although NDV has essentially only one serotype, there is a wide difference in the pathogenicity of different strains, ranging from those that cause virtually no signs to those that kill within a few days. These have been classified in order of increasing pathogenicity, into asymptomatic enteric, lentogenic, mesogenic and velogenic strains. The majority of live vaccines are derived from asymptomatic enteric or lentogenic strains, although some vaccines derived from mesogenic strains are still in use.

Conventional lentogenic vaccines: The level of vaccine reaction is an important consideration for intensive commercial poultry and because HB1 has very mild vaccinal reactions, it has been widely used for initial vaccination of intensive poultry. In a controlled trial in village poultry, HB1 provided effective protection against ND (Bell *et al.*, 1990). La Sota produces moderate vaccinal reactions, especially in immunologically naive birds and is not usually recommended for primary vaccination. In theory, La Sota would also be unsuitable for vaccinating a multi-age population, including young chicks which is inevitably seen in the village situation. This is because the virus spreads and it is not practical to isolate the adults from the chicks. In practice, the degree of reaction from La Sota as a primary vaccine depends on the residual level of antibodies, which could protect the birds from vaccinal reactions, and on the extent of other concurrent infections, such as *Mycoplasma* spp, pathogenic *E. coli*, or infectious bursal disease virus and other respiratory viruses. In intensive systems, vaccination using spray delivery systems which produce small particle sizes, may also exacerbate the vaccine reaction.

Some lentogenic vaccines have been cloned by taking a single infectious virus and growing a homogenous population from it, with the aim of selecting a virus which gives less vaccinal reactions than a La Sota-like virus, while retaining its superior immunogenicity compared to a HB1-like virus. An example of this kind of vaccine is "clone 30".

All conventional live vaccines have the disadvantage of needing to be kept at low temperatures to maintain their efficacy. This is not a problem for intensive poultry production in an industrial setting, but the maintenance of the "cold chain" during distribution can be very difficult in village settings, particularly where there is high ambient temperature.

Another problem that is often encountered when using commercial vaccines in the village situation is that they are sold in vials containing 1 000 or 500 doses, many more than the average village farmer needs. In fact, the packaging is a major component of the cost of manufacturing them, because a vial containing a smaller number of doses would not necessarily reduce the cost proportionally (OIE, 2008).

Oil adjuvant, normally used with inactivated vaccines to improve immunogencicity, has also been tested with live vaccines and found to improve immunogenicity (Peleg *et al.*, 1993), but this combination has not been tested with village chickens.

Heat tolerant vaccines: Some asymptomatic enteric viruses have been noted for their greater heat resistance than more conventional lentogenic viruses. This property has been enhanced by selection and cloning in the laboratory to produce heat tolerant vaccines. These have a distinct advantage in the village situation because it is possible to transport the vaccine without a cold chain. The most extensively used vaccine has been the NDV4-HR vaccine, which was pioneered in Malaysia, where a significant proportion of the village poultry was eventually covered by this vaccine (Ibrahim *et al.*, 1992). The application was in feed, which, because of its thermostability, it was possible to pre-coat with the vaccine. The advantage of this method is that it is not necessary to catch the chickens before vaccinating them. The same vaccine has also been tried in other countries in South East Asia, but not always with the same success as in Malaysia. Tests of its application on a variety of foodstuffs have produced variable results (Spradbrow, 1992). The vaccine was also tested in some African countries, but applied by eye-drop and gave good protection against the virulent virus (Saglid and Spalatin, 1982; Bell *et al.*, 1995). Given the difference between African and Asian feeds, the variety of feeds within Africa, and the variable results with some feedstuffs in Asia, it seems that application of this type of vaccine is best done by eye-drop. It can also be argued that the additional security provided by the vaccine is an incentive to invest in some form of housing, in which case catching the chickens is no longer a problem.

More recently, a similar vaccine to NDV4-HR, called I-2 (Bensink and Spradbrow 1999), has been made available for local production in non-industrialised countries, which has the significant advantage of low cost. In trials in Ghana, Mozambique, Tanzania and Vietnam village chickens, vaccinated with strain I-2, were protected against artificial and field challenge with virulent virus (Amakye-Anim *et al.*, 2000; Dias *et al.* 2001; Tu *et al.*, 1998; Wambura *et al.*, 2000).

Mesogenic vaccines: Mesogenic strains have long been used for vaccination in the village situation. These produce severe vaccinal reactions in an immunologically naïve population, and the use of this kind of vaccine is not advisable in situations where chickens are without any immune protection against the virus. Normally mesogenic vaccines, such as Komarov (Saifuddin *et al.*, 1990) and Mukteswar (Alexander, 1997) are used as secondary vaccines after a primary vaccination with a lentogenic vaccine.

Recombinant vaccines: NDV has two surface glycoproteins, fusion [F] and haemagglutinin/neuraminidase [HN]. The genes coding for either of these can be inserted into a different kind of virus to make a recombinant vaccine. For example, the fusion gene inserted in herpes virus of turkeys produced a vaccine which gave good protection against virulent NDV (Morgan *et al.*, 1993). One advantage of this technique is that the host virus may have better stability than NDV. Another advantage is that antigens for multiple different pathogens can be inserted into the same host virus to produce a single vaccine against several different diseases. Perhaps the most significant advantage for field use is that it is possible to monitor the response to the vaccine independently of the wild virus but in its presence, and conversely, it is possible to detect antibodies against the wild virus in the presence of vaccination. This is done by using an enzyme-linked immunoabsorbent assay (ELISA) that uses a purified antigen, and comparing the results with those of an ELISA using a whole virus antigen. For example, Makkay *et al.* (1999)

prepared an ELISA using only nucleocapsid protein of NDV as antigen. This detected antibodies against wild virus, but not antibodies against a recombinant fowl pox virus expressing HN glycoprotein. A parallel ELISA using whole virus as antigen detected antibodies against the vaccine.

A disadvantage of recombinant vaccines is that where they have been developed commercially the cost is high.

2.8. Status of the disease in Ethiopia

There is no clear record about the introduction of virus to the country. However, Hanson (1978) indicated (reviewing available litrature predated to the year 1926)that ND first occured in and arround sea ports of countries, and spread to the interior of the country along transport routes. The first documented outbreak of Newcastle disease in Ethiopia dates back to 1971 and reported from a small poultry farm in Asmara, Eritrea (NVI, 1974), located close to a sea port and the then province of the country. The first NDV reported was a velogenic type, which was classified according to the virulence of the strain and caused about 80% mortality. In the following years the disease spreads fast to other parts of the country. In 1972, outbreaks had been reported in Addis Ababa (1020km from Asmara) and in 1974 at the then Alemaya Collage of Agriculture poultry farm (1520km from Asmara) (NVI, 1974).

In Ethiopia, some survey works were carried out at varous time to identify constraints of village poultry production under traditional managment system. The result indicated that disease is the most important limitation followed by predators, external parasites and feed shortage. Some farmers have even give up rearing poultry because of increasing disease problem (Edward, 1992; Dessie, 1996). These studies indicated that ND is the major disease problem in all the study areas. This fact is substantiated by Nassir (1998) who reported that in 1995, ND outbreaks in the surounding areas of Debre-Zeit, Nazreth and Addis Ababa killed almost 50% of the local birds.

A study conducted on 180 chickens raised under a traditional managment system in three selected agroclimatic zone reaveled that there were an over all seroprevalce of 32.2 % . Seroprevalce rates of NDV were 28.57%, 29,69% and 38.33% in the high, mid and low altitude respectively. This study has also shown that ND is the major infectous disease threating the survival and productivity of traditionally managed local chickens in central Ethiopia (Taddese *et al.,* 2005).

Another seroprevalence study in village chickens conducted on non vaccinated chickens raised under traditional back yard system revealed that there were an over all seroprevalece rates of 19.78% (n = 280). The study covered dry and wet district. 22.51% (n = 191) seropositive chickens were found in the dry areas of rift valley regions while 14.13% (n = 92) were positive in wet areas of southern regions (Zeleke *et al.,* 2005).

Viruses of Newcastle disease have been isolated from different areas at different times at NVI which were responsible for outbreak cases of the disease (Table 1). It was confirmed that all these isolates were velogenic strain (Nassir, 1998).

Table 1. Origin and year of collection of Necastle disease field virus isolate

No	Sample Id	Place of origin	Year of collection
1	Lab	Koka	1997
2	Walyta	Maji	1997
3	Dembi 1	Debre-zeit	1987
4	Dembi 2	Debra-zeit	1996-1997
5	Lemlem	Debrezeit	1996
6	Kebele 2	Debre-zeit	1984
7	Alamya	Hara	1984
8	Markrt	Arsi	1997

Source: (Nassir, 1998).

A retrospective study conducted from 1983 to 1995 at three state owned poultry farms of Ethiopia showed that nine newcastle disease outbreak had occured. Out of the total of 1,063,699 chickens 621,721 (58%) were affected and among these, 92,429(14.9%) were died during the outbreaks (Nassir, 1998).

2.9. Public health importance

Newcastle disease viruses, whether virulent field viruses or live vaccine, can produce a transitory conjunctivitis in humans; the condition has been limited primarily to laboratory workers and vaccination teams exposed to large quantities of virus, before vaccination was widely practiced, in workers eviscerating poultry in processing plants. The disease has not been reported in individual who rear poultry or consume poultry products (Alexander, 1997).

Newcastle disease virus (NDV) is a virus that is of interest because it replicates (makes copies of itself) more quickly in human cancer cells than in most normal human cells and because it can kill these host cells NDV can be used to directly kill cancer cells, or it can be given as a cancer vaccine. Cancer vaccines cause the body's natural immune system to seek out and destroy cancer cells. However, the US Food and Drug Administration has not approved NDV as a treatment for cancer.

3. MATERIALS AND METHODS

3.1. Study area

The study was conducted in Southwest Shewa zone, Kersana Kondaltiti woreda. The woreda is one of the 180 woredas in the Oromia Region of Ethiopia. It is bordered in the south by the Southern Nations, Nationalities and Peoples Region, in the west by Kokir, in the northwest by Tole, in the northeast by Alem Gena, and on the east by the Misraq Shewa Zone. It is located at the boarder between the two regions, Oromia and Southern Nations, Nationalities and Peoples Region. The major town in Kersana Kondaltiti is Leman.

The altitude of this woreda ranges from 1500 to 2900 meters above sea level. The area is located $38^0$3'E and $9^0$3'N. It is characterized by mild subtropical whether, with maximum and minimum temperature ranging from 2^0c to 9^0c and 20^0c to 27^oc respectively. This area experiences a binominal rainfall pattern with a long rainy season from June to September and short rainy season from March to April.

Based on figures published by the Central Statistical Agency in 2005, this woreda has an estimated total population of 127,704, of whom 63,442 were males and 64,262 were females; 3,534 or 2.77% of its population are urban dwellers, which is less than the Zone average of 12.3%. With an estimated area of 975.94 square kilometers, Kersana Kondaltiti has an estimated population density of 130.9 people per square kilometer, which is less than the Zone average of 152.8 (CSA, 2008).

3.2. Poultry population and management system in the area

The most recent estimate of chicken population in the woreda is 64,395 (CSA, 2008). The production system in the area is characterized by minimum input from the owners; with chickens scavenge around the farm or belonging to households using no major inputs other than occasional grain feeds and household wastes. The standard of housing varies greatly. In some areas primitive poultry houses are built from simple locally available materials and in most areas the chickens share with humans. Fertile eggs are hatched under brood hen and it attends the clutches of chicks. Meat and egg production by the local chickens in the area is very low. Much of the chickens in the worda are local breads but there are also cross breeds especially in low land areas of the study sites. The population of poultry in highland is very low and almost all of them are local. This is due to the extensive production of "enset" in the highland area since chickens eat the plant, farmers do not want to produce poultry. There is no history of vaccination program in the woreda.

3.3. Study design, sample size and sampling methodology

The approach followed to determine sample size and design for the prevalence study was the multistage cluster sampling because the individual sampling frame was not known. Thus, samples of clusters were selected, followed by sub sampling of some chickens in the clusters.

Owing to the characteristics of the back yard system and the nature of the disease, it is reasonable to consider all the back yard poultry in the PA as one flock, with very little within cluster variation, so PA (Peasant Association) was considered as a cluster, which was the primary unit, house holds are the secondary units and the selected members (each member selected i.e. chickens) of the sub samples were the tertiary unit.

The relevant formula to determine the sample size for simple random sampling for a 95% confidence interval is:

$$n = \frac{1.96^2 P_{exp} (1-P_{exp})}{d^2}$$

Where, n = required sample size, P_{exp} = expected prevalence , d = desired absolute precision (Thrustfield, 2005).

A seroprevalence study of Newcastle disease, which was conducted in village chickens in Butajira area, which is the border of the study area, showed that there was a prevalence of 10% (Zeleke *et al.,* 2005). Therefore, by taking the expected prevalence of the study areas as 10%, the appropriate sample size would be 138.

According to Martin *et al.,* (1987), it is possible to apply the formula used for calculating sample size in simple random sampling and then multiplying the estimated sample size by two for cluster sampling. So to obtain similar accuracy to that with simple random sampling (138 using 10%

expected prevalence, 5% accuracy and 95% confidence interval), the sample size had to be increased two times, to 276.

There were 32 clusters (PA) in the study area (wereda). We took 8 clusters (PA) randomly(25%), three from high land and six from low land area of the study sites. The aim was to sample 3% of the total chicken population from each cluster, resulting in 355 samples allowing around 28.6% more samples than required that increase the precision from 98 households.

3.4. Samples for virus isolation

Samples were collected from apparently healthy chicken and outbreak case in the study area according to the protocol of OIE terrestrial manual, (2008). From recently dead chickens, we took tracheal and cloacal swab which were collected separately as well as samples collected from lung, kidney, intestine (including contents), gizzard, proventriculus, spleen, brain, liver and heart tissues. These were collected as pool, although intestinal, gizzard, proventriculus, and liver samples were taken and processed separately from other samples. The samples were put into labeled universal bottles containing phosphate buffered saline, pH 7.0-7.4, and containing antibiotics.

Tracheal and cloacal swabs were taken from apparently healthy chickens. The later were visibly coated with faecal materials. Then the samples were placed in a labled cryovials having isotonic phosphate buffered saline (PBS), pH 7.0-7.4, and containing antibiotics. Finally, the samples were kept cool during transport to the laboratory, where they were frozen at -80°c until the isolation was carried out.

3.5. Virus culture

Virus culture or virus isolation was performed following the protocol of OIE terrestrial manual, (2008). The supernatant fluids of swabs or homoginised tissue suspensions, obtained through clarification by centrifugation at 1000 g for 10 minutes, were inoculated in 0.2ml volumes in to the allantoic cavity of three embryonating eggs of 9-11 days incubation. After inoculation, the eggs were incubated at $37^{0}C$ for five days. The eggs were candled every 24 hours. Eggs containing dead embryos, and all eggs remaining at the end of the incubation period, were chilled at $+4^{0}C$ and allantoic fluids were harvested and tested for its ability to haemagglutinate chicken RBCs. Fluids that gave a negative reaction were passed in to one further batch of eggs.

3.6. Virus identification

For identification of viruses, haemagglutination and haemagglutination inhibition tests were conducted on the allantoic harvest based on the recommendations of the OIE terrestrial manual, (2008). A 0.025ml of PBS was dispended in to each well of a plastic V-bottomed microtiter plate and the same amount of the virus suspension (i.e infective allantoic fluid) were placed in the first well. After that twofold dilution of 0.025ml volumes of the virus suspension were made across the plate. Then a further 0.025ml of PBS was dispensed to each well to make the final volume 0.075ml after dispensing 0.025ml of 1%(v/v) chicken RBCs to each well. The solutions were mixed by tapping the plates gently. The RBCs were allowed to settle for about 40 minutes at room temperature when controls RBCs were settled to a distinct button. Haemagglutination was determined by tilting the plate and observing the presence or absence of tear shaped streaming of the RBCs. The titration was read to the highest dilution giving complete HA (no streaming); this represents 1 HA units (1HAU).

Haemagglutination activity detected in bacteriologically sterile fluids harvested from inoculated eggs was confirmed by the use of specific antiserum for Newcastle disease virus in a haemagglutination inhibition test based on again OIE terrestrial manual, (2008).

3.7. Extraction of RNA from allantoic harvest

GenElute Mammalian Total RNA Kit was used for the extraction of RNA from the allantioc harvest based on the protocol of the kit. A 500μl of lysis solution and 5μl mercaptoethanol was added in to the vessel containing the allantoic harvest (140 μl). Then lysate was transferred to filtration column and centrifuged at 14,000 g for 2 minutes which was followed by adding 500μl ethanol (70%) to the filtrate, load the lysate in to binding column, pipette up to 700μl of the lysate /ethanol mixture in to a genelute binding column, centrifuge at 14,000 g for 15 seconds, and discard the flow-through liquid. The next step is to remove the contaminants by washing. A 500μl of wash solution 1 was pipetted in to the column and centrifuged at 14,000 g for 15 seconds, transfer the binding column into a new collection tube, pipette 500μl of wash solution 2 in to the column and centrifuge at 14,000 g for 15 seconds, discard the flow through, pipette a second 500μl volume of wash solution 2 in to the column and centrifuge at 14,000 g for 2 minutes. Finally, the binding column was transferred to a new collection tube, 50μl of the elution solution was pipetted in to the center of the binding column and centrifuged at a maximum speed for 1 minute. Then the RNA extract was stored at a temperature of -20^{O}C until the real time RT-PCR was carried out.

3.8. Real time RT-PCR

QIAGEN one-step RT-PCR Kit was used for Reverse transcription PCR. Reverse transcription PCR was carried out sequentially in the same tube. All components required for both reactions were added during setup, and there was no need to add additional components once the reaction has been started. Optimal reaction conditions, such as incubation times and temperatures during PCR amplification, will vary and need to be determined individually. The master mix was prepared according to the protocol of the kit which was as follows:

Thaw template RNA, primer solutions, dNTP Mix, 5x QIAGEN OneStep RT-PCR buffer, and RNase-free water, probe, enzyme mix and place them on ice. Their reaction volume or concentrations were according to the prescriptions of the kit. Mix thoroughly, and dispense appropriate volumes into PCR tubes. Add template RNA to the individual PCR tubes. Program the thermal cycler according to the program outlined below.

A reverse-transcription reaction temperature of 50°C for 30 minutes, Taq DNA Polymerase was activated at 95°c for 15 minutes, Reverse Transcriptase were inactivated and the cDNA template was denatured at this step. Finally, we set denaturation at 94°C for 10 seconds, annealing at 56°C for 30 seconds and extension at 72°C for 10 seconds. Number of cycles are 40. Start the RT-PCR program while PCR tubes are still on ice. Wait until the thermal cycler has reached 50°C. Then place the PCR tubes in the thermal cycler. The thermal cycler used in our lab was platform miniopticon and the software for reading was opticon monitor 3.

3.9. Serological test

3.9.1. Sera collection

Approximately 2 ml of blood was collected from the brachial vein of chickens using a 3ml syringe and a 23-gauge needle after disinfecting the site. The whole blood collected from local chickens was labeled and allowed to clot under normal atmospheric condition within the syringe. Then the clear serum was harvested into labeled cryovials and stored at -20 ^{0}C until the ELISA test was carried out.

3.9.2. Enzyme Linked Immunosorbent Assay

The SVANOVIR® Newcastle disease antibodies ELISA kit was used according to manufacturer procedure (svanova Biotech AB, Uppsala, Sweden) to detect NDV specific antibodies in the serum samples. The kit procedure (Annex 3) is based on the blocking enzyme linked immunosorbent assay (Blocking –ELISA). In this procedure, serum samples were exposed to non infectious NDV antigen coated wells in microtitere plates. If antibodies directed to NDV were absent in the samples these sites would remain free. When horseradish peroxidase (HRP) conjugated monoclonal antibodies directed to NDV are added, they will bind to these free sites on the virus. After addition of the substrate a blue color developed. A negative result was indicated by a strong color change. The reaction was stopped by the addition of the stop solution; the color changed to yellow. The result was read by a microplate photometer, where the optical density (OD) is measured at 450nm.OD readings from the samples was compared to those of the positive and negative control sera.

To ensure assay validity, the negative control should have an optical density (OD) value greater than 0.600.The positive control should have a PI (percent inhibition) =

$$\frac{\text{(OD Neg. ctrl- OD Pos.ctrl)}}{\text{ODNeg.ctrl}}\text{X100, value greater than 40.}$$

Sample shaving PI value greater than 40, 30-40 and < 30 were positive, doubtful and negative respectively. Samples with a PI value between 30 and 40 were considered doubtful and were retested.

3.10. Data Analysis

Data entry was done using Microsoft office Excel and processed using SPSS version 15 statistical soft ware after importing the data from microsoft excel. Descriptive statistics were computed for all the parameters and different kebeles. Chi square and Fischer's Exact tests was used to analyze the differences in the seroprevalence between the sexes, ages, altitudes and breeds. A P-value less than 0.05 were considered to be statistically significant.

4. RESULTS

Out of the 355 sera from the chickens sampled, 5.6% were positive with ELISA to Newcastle disease virus. The over all seroprevalence of Newcastle disease virus antibodies in this study area were therefore 5.6% (3.2__8.0 % prevalence at 95% CI). Five (62.55%) of the eight Kebeles sampled had chickens that were positive for antibodies against Newcastle disease virus by the blocking ELISA. The prevalences in each Kebele are presented in Table 2 and 3.

Table 2. Seroprevalence of Newcastle disease virus antibodies in lowland Kebeles

Kebele	Positive sera	Negative sera	Total	Prevalence
Kersa	0	27	27	0
Wenber	3	68	71	4.2
Adadi	3	65	68	4.4
Muti	4	41	45	8.9
Harbu	9	23	32	28.1
Total	19	224	243	7.8

There prevalence ranges from 0 to 28.1% in the lowland kebeles and the overall seroprevalence of the district was 7.8% (4.43% to 11.7 % at 95% CI). Only one (20%) of the kebeles sampled had no positive samples for newcastle disease virus antibodies. The rest 80% of the kebeles sampled do have positive serum for newcastle disease virus antibodies. The highest prevalence (28.1%) of Newcastle disease virus antibodies was recorded at Harbu Kebele followed by Muti, Adadi and Wenber Kebeles(Table 2).

Table 3. Seroprevalence of Newcastle disease virus antibodies in Highland Kebeles

Kebele	Posetive sera	Neggative sera	Total	Prevalence
Ilala	0	55	55	0
Wako	0	19	19	0
Godeti	1	37	38	2.6
Total	1	111	112	0.9

Only one kebele (33.3%) from the highland had seropositive animal but the rest two kebeles (66.6%) did not have positive chickens for Newcastle disease virus antibodies in their serum. The overall seroprevalence in lowland area was only 0.9 % (0 to 2.64 % at 95 CI) (Table 3).

Table 4. Description of study chickens by altitude, age, sex and breed

	Altitude		Age		Sex		Breed	
	Highland	Lowland	Young (3-6 months)	Adult (> 6 months)	Male	Female	Local	Cross
Pos sera	1	19	0	20	1	19	8	12
Neg sera	111 (99.11)	224 (92.2)	81 (100)	254 (92.8)	50 (98.1)	285 (93.8)	288 (97.3)	47 (79.7)
Total	112	243	81	274	51	304	296	59
Prevalence	0.9	7.8	0	7.2	1.9	6.2	2.7	20.3
χ2-value	6.92		6.26		1.51		28.78	
Significance	p = 0.009		p=0.01		p=0.331		p=0.00	

A significant ($p<0.05$) difference in the prevalence of Newcastle disease virus antibodies was found between highland and lowland areas, adult and young chickens ($p< 0.05$). Furthermore, there was a highly significant difference ($p = 0.00$) in the NDV antibodies level between local and cross breeds of chickens (Table 4).

The difference, however, is not statistically significant ($p>0.05$) for sexes (Table 4). Highest prevalences were recorded in cross breeds of chickens (20.3%) and sero-positive samples were not found in the young chickens. Only one sero-positive sample was found in the highlands of the study site (Table 3 and 4).

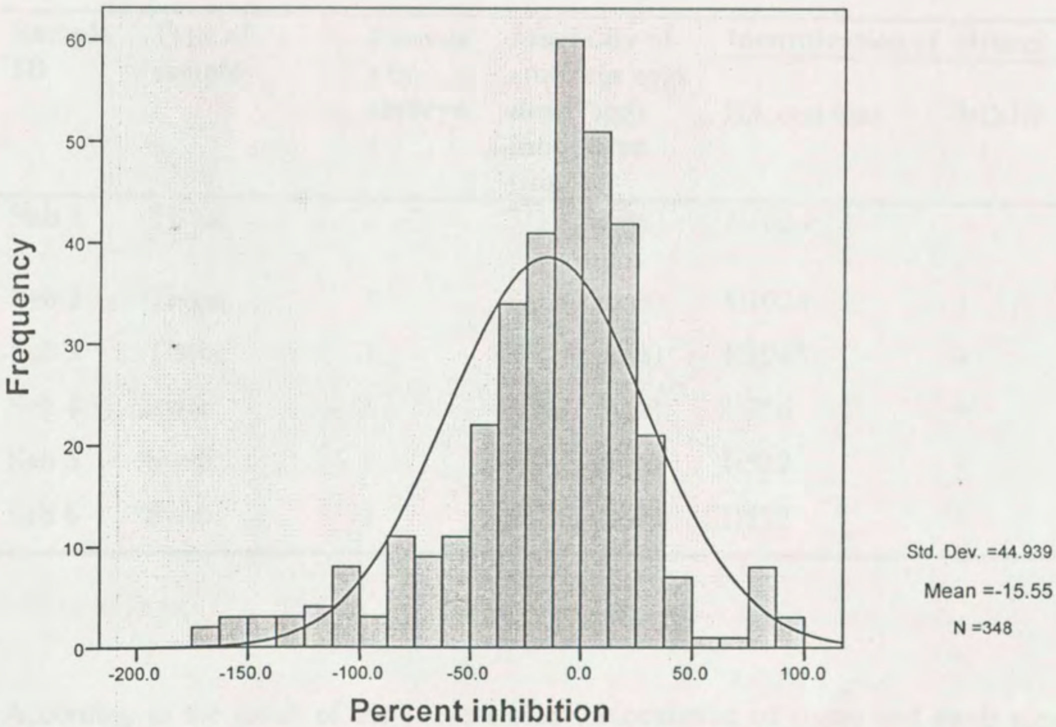

Figure 1. Distribution of the percent inhibition values of the chicken sera

This figure indicted that sixty eight percent of the chickens sampled do have a Percent Inhibition (PI) value of between -60.49 to 29.3 and much of (95%) of samples do have a PI value between -105.43 and 74.33 (Fig 1). The percent inhibition values of all the chicken sera are almost normally distributed.

From a NDV suspected outbreak areas six samples were collected from dead and sick chicken for isolation and identification of the Newcastle disease virus (Table 5).

Table 5. Isolation and identification of Newcastle disease virus from out break areas of suspected ND cases

Sample ID	**Type of samples**	**Passages in embryos**	**Mortality of embryos eggs dead /eggs inoculated (time)**	**Identification of viruses**	
				HA end titer	ND/HI
Seb 1	Tissue	1	3/3 (2 days)	1:1024	+
Seb 2	Tissue	1	3/3 (2 days)	1:1024	+
Seb 3	Tissue	1	3/3 (2 days)	1:2048	+
Seb 4	Swab	1	3/3 (2 days)	1:256	+
Seb 5	Swab	1	3/3 (2 days)	1:512	+
Seb 6	Swab	1	3/3 (2 days)	1:512	+

According to the result of the present study, inoculation of tissue and swab suspension from recently dead and sick chickens resulted in the death of all embryos within 48 hrs. Allantoic harvest from the dead embryos agglutinated chicken erythrocytes with a different HA titer (Table 5), which were subsequently inhibited by NDV antiserum. This result indicated that Newcastle disease virus was isolated from all (100%) six tissue and swab suspensions.

But cloacal and tracheal swab samples taken from 30 apparently healthy chicken revealed that, there is no haemagglutinating viral agents. None of the samples can lead to the death of the embryo even at the second passage.

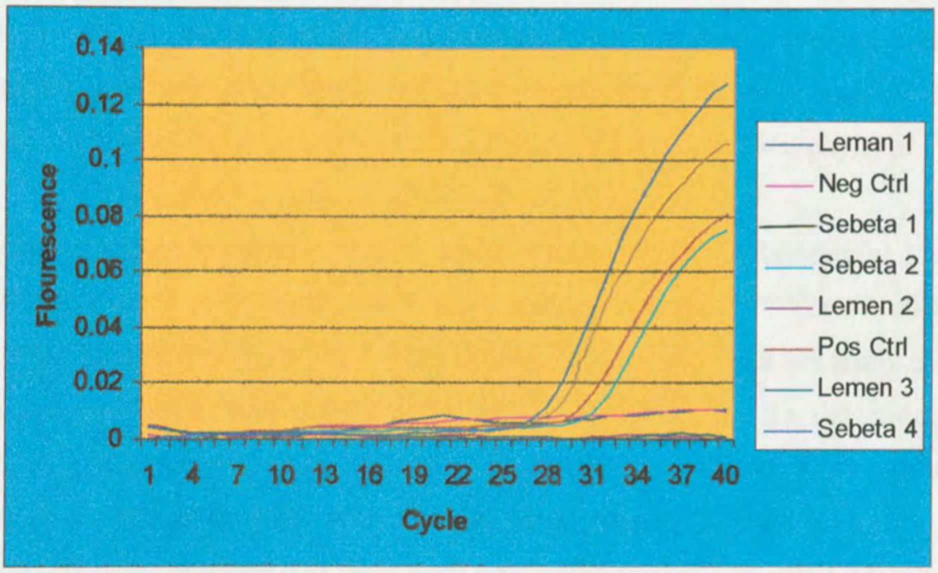

Figure 2. Real time RT-PCR results of six samples taken from suspected cases of out break area and apparently healthy chickens

Subjection of genomic RNA to real time RT-PCR using specific primer for fusion protein cleavage site (FPCS) resulted in the amplification viral genome (Fig. 2). Three of samples taken from out break cases were amplified but none from apparently healthy chicken. All the three samples, which were positive by HA, and HI were also positive by real time RT-PCR. Again, all the three samples negative for HA and HI were also negative for real time RT-PCR. There is 100% test agreement between HI and real time RT-PCR in this case.

5. DISCUSSION

Antibodies to NDV were identified in five (62.55%) of the eight Kebeles sampled, which agrees with similar studies in Botswana, Uganda, Mexico and Ethiopia (Zeleke *et al.,* 2005; Gutierrez-Ruiz *et al.,* 2000; Mukiibi-Muka and Olaho-Mukani 1998; Mushi *et al.,* 2001). The presence of Newcastle disease virus antibodies in the sera of the chicken in this study was an indication of previous exposure of the chickens to the virus. Since there is no history of vaccination in the study area, the chickens might have been exposed to natural infection. Since all of the chickens sampled were over three months of age the presence of maternal antibodies can be ruled out for such antibodies are known to wan after the age of 3-4 weeks (Murphy *et al*., 1999).

Zeleke *et al.,* 2005 reported an over all prevalence of NDV antibodies as 19.78% in southern and rift valley areas of Ethiopia. The overall seroprevalence of NDV antibodies 5.6% (95% CI 3.2-8.0%) found in this study were lower than the above study, but the study methods were different in that they used Haemagglutination Inhibition method for the detection of serum antibodies where as ELISA was used for this study for the detection of NDV antibodies. A serological survey conducted for NDV antibodies in village chickens in Mexico having similar study design with this study revealed that there were seroprevalence of 2.2% (95 CI 0.5-3.8%) (Gutierrez-Ruiz *et al.,* 2000), which agrees with this study.

The low prevalence or absence of detectable antibodies to NDV in the four kebeles sampled indicated that the village chickens in such kebeles would be highly susceptible to the pathogenic NDV infection (Allen and Gough, 1974). In their serological study in Uganda, Mukiibi-Muka and Olaho-Mukani (1998) found that the birds from one village out of the three sampled did not have antibodies to NDV. Similar finding is reported by Zeleke *et al.,* 2005 which stated that chickens from two out of seven districts of southern and rift valley region of Ethiopia did not have positive sera for NDV antibodies; which indicated that, even in enzootic areas, some village can remain fully susceptible to infection with virulent NDV.

The prevalence varied between sexes; however, the difference was not statistically significant. This finding of the study is in accordance with the previous studies of Ethiopia in showing that there no significant difference between the two sex groups (Zeleke *et al.*, 2005; Taddese *et al.*, 2005).

In this study, there was a significant difference ($p<0.05$) in the sero prevalences between altitudes. The low altitudes do have higher sero prevalence than the high altitude which agrees with the findings of Zeleke *et al.,* 2005 and Tadesse *et al.,* 2005 who reported that there were a high prevalence in the lowland than high land even though the difference is not significant in their study. The possible explanation for this could be there are few chickens in the high land area of the study sites and chicken population number is a factor for the transmission of the disease.

The observed differences in the rates of NDV ELISA antibodies in the two districts may also be because of ecological variations in ND activity and may perhaps be a reflection of the impact of environment on the viability of NDV and epidemiology (Orajaka *et al.*, 1999). Other factors responsible for differences in NDV ELISA antibody may be as result of the presence of commercial poultry farms in the lowland. There is no poultry farm in the highland but there is one poultry farm in the lowland. Commercial poultry are routinely vaccinated against ND virus but rural household chicken may come in contact with them especially during sales. Hadipour, (2009) demonstrated that the risk of back yard chicken flock being seropositive for NDV increased with the increasing the proximity to the nearest neighbor poultry farm.

The difference in the seroprevalence between > 6 months and 3-6 months was also statistically significant ($p<0.05$), which agrees with the finding of (Vui *et al.,* 2002) which stated that in the 2- 6 months-old groups had a significantly lower NDV antibody titre than the > 6month-old age group. This can be hypothesized to be due to more frequent exposure of older birds to field virus, which might have survived the disease at an earlier age.

A significant difference (p=0.00) in the seroprevalence between the lacol and cross breeds of chickns has been found in the study area which disagrees with the finding of (Vui *et al.,* 2002). They found that there was no significant difference between the local and hybrid chicken. The question of breed susceptibility to ND is still controversial (Awan *et al*., 1994).The possible explanation of this finding is that it may be from vaccine source though the farmers said that there was no vaccination program in the woreda. Hemagglutination inhibiting antibody can be detected within 4 to 6 days of infection and persists for at least 2 years (Murphy *et al.,* 1999).

Highest prevalence (28%) was recorded at Harbu kebele, which is located just near to the market. The proximity of the kebele to the market may be the reason for the highest prevalence of the disease in this kebele.

Fig 1 showed that distribution of percent inhibition of chicken sera where the percent inhibition values were distributed normally with the majority (95% of the PI values) lies between -105.43 and 74.33. This finding was in the agreement with Schelling *et al.,* (1999), which showed that there were normally distributed values of the percent inhibition values of the bird serum.

Eggs cultured later on HA and HI tests carried out at the National Animal Health and Disease Investigation Center, Ethiopia (NAHDIC) confirmed that all the isolates were avian paramyxo virus type 1(APMV-1) serotype. The fusion protein cleavage site of the isolate was amplified with real time RT-PCR, further confirmed the identity of the isolate as avian paramyxovirus type 1. Nasir, (1998) had also isolated and identified avian paramyxovirus type 1 as the causes of outbreaks of suspected Newcastle disease. This finding indicated that the disease is endemic in the study area.

The time of outbreak in the study site was at Christmas time. Sa'idu *et al*. (2006) and Nwanta *et al*. (2006) reported that increased movement of sick and healthy chickens in anticipation of various festivals particularly of Christmas and New Year. These may have been responsible for the occurrence of ND outbreak in January in the study area.

6. CONCLUSIONS AND RECOMMENDATIONS

Newcastle disease virus (Avian paramyxovirus type 1) was isolated from outbreak cases of chicken in the study area. The low prevalence or absence of detectable antibodies to NDV in the four kebeles sampled indicated that the village chickens in such kebeles would be highly susceptible to the pathogenic NDV infection. Thus, it is recommended that there should be routine vaccination program in the study area.

Highest prevalence (28.1%) was recorded at Harbu kebele, which is located just near to the market. The time of the outbreak at the study site was at Christmas time when there was mass mobilization of chickens to the market. Within the country, NDV may spread with animal movements, such as buying, selling and exchanging poultry. The risk of markets, however, has not been evaluated and thus their role in the spread of NDV is not known. Therefore, further study should be conducted to determine the role of markets for the transmission of the diseases in the country.

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8. ANNEXES

Annex 1. Procedures of ELISA test for the detection of NDV antibodies in chicken serum

1. All reagents should equilibrate to room temperature 18 to 25^{0}C, (64 to 77^{o}F) before use. Label each strip with a number.
2. Add 100 µl of positive control solution (A) and 100 µl of negative control solution (B) to selected wells. For confirmation purpose it is recommended to run the control sera in duplicates.
3. Add 50 µl of PBS –Tween Buffer and 50 µl of undiluted serum to each of the selected wells and mix thoroughly.
4. Seal the plate and incubate for 30 minutes at room temperature 18 to 25^{0}C, (64 to 77^{o}F).
5. Rinse the plates/strips 3 times with of PBS –Tween Buffer: at each rinse cycle fill up the wells, empty the plate and tap hard to remove all remains of fluid.
6. Add 100 µl of HRP conjugate to each well.
7. Seal the plate and incubate for 30 minutes at room temperature 18 to 25^{0}C, (64 to 77^{o}F).
8. Repeat step # 5
9. Add 100µl substrate solution to each well. Incubate for 10 minutes at room temperature 18 to 25^{o}C, (64 to 77^{o}F). Begin timing when the first well is filled.
10. Stop the reaction by adding 50 µl stop solution to each well and mix thoroughly. Add the Stop solution in the same order as the substrate solution in step # 9.
11. Measure the optical density (OD) of the controls and samples at 450 nm in a microplate photometer (use air as blank). Measure the OD within 15 minutes after the addition of stop solution to prevent fluctuation in OD values.

Calculations

1. Calculate the mean OD –values for each of the controls and samples
2. Calculate the percent inhibition (PI) values for positive control as well as samples, using the following formula:

$$PI = \frac{(OD_{Neg\ ctrl} - OD_{Sample/Pos\ ctrl}) \times 100}{OD_{Neg\ ctrl}}$$

Interpretation of the results

Criteria for test validity

To ensure assay validity, the negative control solution should have an optical density (OD) value greater than 0.600. The positive control solution should have a PI greater than 40. For invalid tests, technique may be suspected and the assay should be repeated.

Interpretation

PI > 40	positive
PI 30- 40	doubtful
PI < 30	negative

Samples with a PI between30-40 are considered doubtful and should be retested. If the results are still doubtful, the flock should be sampled and tested again within 10-14 days.

Annex 2. Preparation of 1% chicken red blood cells (RBC)

Procedure

1. RBC'S from a minimum of three specific pathogen free or serum neutral chickens are pooled in an equal volume of Alsever's solution or equivalent anticoagulant (such as heparin, citrate or EDTA).
2. RBC's are washed three times in PBS buffer by centrifugation for 5 minutes at 2000 rpm.
3. A 1% v/v suspension of the washed RBC's is prepared (i.e. 1ml of packed cells to 99ml of PBS)
4. Suspension is kept at +4^{0}C-10^{0}C until needed
5. Fresh 1% RBC suspension should be mace every time the HA/HI test is prepared

Annex 3. Preparation of Alsever's solution

Reagents

Citric acid	0.055g
Sodium Citrate	0.8g
D-Glucose	2.05g
Sodium chloride	0.42g

Distilled water to make up to 100 mL

Method

1. Weigh out reagents into a conical flask.
2. Dissolve of distilled water and make up to100 mL.
3. Dispense into sterile 10 mL bottles.
4. Sterilize by autoclaving at 116°C for 10 minutes.
5. Allow to cool, then tighten the lids and label the bottles.
6. Store in the refrigerator

Annex 4. Equipment and reagents used for the extraction of RNA from Allantoic harvest

- GeneElute mammalian total RNA kit
  - Lysis Solution
  - 2-Mercaptoethanol
  - 70% ethanol
  - Wash Solution 1
  - Wash Solution 2 Concentrate
  - Elution Solution
  - GenElute Filtration Columns in Tubes
  - GenElute Binding Columns in Tubes
  - Collection Tubes

Annex 5. Real time RT-PCR reaction mix volumes for parmyxovirus type 1 (F gene)

Component	Volume/ reaction	Final concentration
Master mix		
RNase-freeH_2O	6.95μl	
25 mM $MgCl_2$	1.25 μl	3.75mM
5x QIAGEN RT-PCR buffer	5 μl	1X
Enzyme mix	1 μl	
Primer F	0.5 μl	10pmol
Primer R	0.5 μl	10pmol
dNTP's	0.8 μl	320μMea.dNTP
Probe	0.5 μl	0.12μM
Rnase inhibitor	0.5 μl	13 units
MM / reaction	17 μl	
Template RNA	8 μl	
Total volume	25 μl	

Annex 6. Real time RT-PCR cycler conditions

Step	Time	Temperature	Remark
Reverse transcription	30 minutes	50 ^{0}C	
PCR initial activation step	15 minutes	95 ^{0}C	Taq DNA polymerase is activated
3- step cycling			
Denaturation	10 seconds	94 ^{0}C	
Annealing	30 seconds	56 ^{0}C	
Extension	10 seconds	72 ^{0}C	
Cycle number	40 cycles		

9. CURRICULUM VITAE

A. PERSONAL INFORMATION

Name:	Belayneh Getachew Ayalew
Sex:	Male
Place of birth	South Wollo/ Mekaneselam
Date of birth:	Oct 5, 1982
Martial status:	Single
Language:	Amharic and English
Religion:	Orthodox/ Christian
Nationality:	Ethiopian

B. EDUCATIONAL BACKGROUND

Elementary:	Legeamhara elementary school, Mekane selam (1988 -1995)
High School:	Borena senior secondary school, Mekane selam (1996 -2000)
University:	Addis Ababa University, Faculty of Veterinary Medicine.

C. EDUCATIONAL QUALIFICATION

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D. WORK EXPERIENCE

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E. THESIS WORK

- DVM: Retrospective study of major Surgical and obstetrical cases at FVM open air clinic, Debre zeit, Ethiopia.
- MSC: Isolation, identification and seroprevalence of Newcastle disease virus in village chicken in south west shewa zone, Ethiopia.

10. SIGNED STATEMENT OF DECLARATION

I, the under signed, declare that this thesis is my original work, has not been presented for a degree in any other university and that all sources of material used for the thesis have been duly acknowledged.

Name: Belayneh Getachew Ayalew

Signature_______________

Date of submission

This thesis has been submitted for examination with my approval as a university advisor

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