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Epidemiology of Newcastle disease in village chickens in Ethiopia: risk factors, molecular characterization and role of poultry markets

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Declaration:

I declare that this thesis is my own account of original research and has not previously been submitted by me for a degree at any tertiary education institution.

Hassen Chaka Chende

November, 2012

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Summary

Village chickens contribute considerably to the economy and to the nutritional requirements and livelihood of many rural farmers in developing countries across the globe. The spread of highly pathogenic avian influenza H5N1 into Africa during 2005/6 drew attention to the neglect of avian disease surveillance and research in countries such as Ethiopia, in which predominantly village chickens are reared. Several infectious and non-infectious diseases have limited the productivity of village chickens in Ethiopia, among which Newcastle disease (ND), caused by avian paramyxovirus serotype 1 (APMV-1), is the most important. Newcastle disease virus (NDV) causes subclinical to severe disease depending on the virus strain. To better understand the epidemiology of the disease, a study was performed in the mid-Rift Valley area of Oromia region, Ethiopia, to estimate seroprevalence and incidence of NDV exposure, identify risk factors, evaluate market trade movements and characterize circulating NDV strains.

Repeated serological surveys in live bird markets revealed that village chickens were concurrently seropositive for several important infectious diseases, particularly during the wet season. The seroprevalence of ND, *Pasteurella multocida* infection, *Mycoplasma gallisepticum* infection and infectious bursal disease virus infection were 5.9%, 66.2%, 57.7% and 91.9%, respectively, during the dry season, and 6.0%, 63.4%, 78.7% and 96.3%, respectively, during the wet season. This underlines the need for a holistic approach to control of infectious disease in village chickens, and further studies are warranted to better understand the circulating strains, their interactions and their economic effect on village poultry production.

A cross-sectional study using a multistage random sampling design with repeated sampling periods was done in households, along with a structured questionnaire. The prevalence of household flocks with at least one seropositive chicken was higher during the dry season (27.4%) than during the wet season (17.4%) (P = 0.003) while the proportion of flocks in which viral genome was detected was 24.2% and 14.2 %, respectively. The prevalence of NDV genome detection in individual birds at markets varied from 4.9 % to 38.2, depending on the period of sampling and the reverse transcriptase polymerase chain reaction (RT-PCR) technique employed. Multilevel mixed-effect logistic regression models were used to identify risk factors for NDV seropositivity and for incidence of NDV exposure. Reduced frequency

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of cleaning of poultry waste, larger flock size and use of an open water source (pond or river) for poultry were associated with increased risk of NDV exposure or seropositivity, while maintaining a closed flock and the use of a grain supplement was associated with lower odds of seropositivity or a lower risk of NDV exposure.

Molecular characterization and phylogenetic analysis, based on complete F and HN gene sequencing, was done on NDV isolates obtained at markets and villages. The circulating viruses had amino acid motifs characteristic of virulent strains, indicating endemic circulation of virulent virus in village chickens which poses a threat to improvement of village chicken production and emerging small-scale commercial poultry production. The strains clustered in genotype VI, branching with viruses from subgenotype VIb that commonly affect pigeons, although clustering apart on pairwise distance analysis. The apparent poor biosecurity in village chickens and history of isolation of pigeon variant viruses from domestic chickens in Ethiopia suggest that pigeons could play a role in the epidemiology of ND in village chickens. Further surveillance and virus characterization is required to shed more light on this.

Bayesian methods were used to evaluate the performance of two commercial enzyme-linked immunosorbent assay (ELISA) kits (a blocking and an indirect ELISA) and haemagglutination inhibition (HI), in the absence of a gold standard, for their ability to detect antibodies to NDV in chicken serum from villages and live bird markets. The blocking ELISA had the highest sensitivity (*Se*) of 96.3% (95% posterior credible interval (PCI): 88.1; 99.8%), and specificity (*Sp*) of 98.9% (95% PCI: 97.8; 99.9%), while the HI had *Se* of 81.6% (95% PCI: 71.8, 91.9%), and *Sp* of 96.1% (95% PCI: 95.1; 96.6%). The indirect ELISA also had high *Se* (95.2%; 95% PCI: 88.5; 99.0%) but had very low *Sp* (8.9%; 95% PCI: 6.4, 11.8%). There is therefore a need for evaluation of commercial kits before their wider use in village chickens under field conditions.

Market trade movement patterns for live chickens were described, using social network analysis, for two different periods during the year 2010, representing high (period one) and low (period two) seasons for poultry trade. The study revealed that the networks exhibited scale-free characteristics with weak connectivity of the markets and low density of the networks. The density for the two periods was not difference (P = 0.29), although a somewhat higher number of markets and links were observed during period one than period two. The low density of the networks indicates that in the event of infectious disease outbreaks in surroundings of the respective markets, the risk of its spread to many others

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would likely be fairly low. Nevertheless, the close similarity of NDV isolates from distant markets in the study area suggests that markets could play a role in the spread of infectious poultry diseases. A few markets were more central in the networks, in terms of their betweenness and out-degree; these markets could be considered for targeted surveillance, while those markets with high in-degree, mainly situated in the larger urban centres, can be considered for surveillance that involves regular poultry traders.

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List of abbreviations

Abs	Antibodies
ACIAR	Australian Centre for International Agricultural Research
AI	Avian influenza
AIDS	Acquired Immune Deficiency Syndrome
APMV	Avian Paramyxovirus
ARC-OVI	Agricultural Research Council - Onderstepoort Veterinary Institute
ATJK	Adami-Tulu-Jido-Konbolcha
AusAID	Australian Agency for International Development
BDSL	Biological Diagnostic Supply Limited
bp	Base pairs
cDNA	Complementary DNA
CIRAD	Coopération Internationale en Recherche Agronomique pour le Développement
CRD	Chronic respiratory disease
CSA	Central Statistical Agency
DIC	Deviance information criterion
DNA	Deoxyribonucleic acid
dpi	Days post-infection
ELISA	Enzyme-Linked Immunosorbent Assay
F	Fusion gene
FAO	Food and Agricultural Organization of the United Nations
HA	Haemagglutination
HAU	Haemaggluting Units
HI	Haemagglutination inhibition
HN	Haemagglutinin-Neuramindase
HPAI	Highly pathogenic avian influenza
HRP	Horseradish Peroxidise
IBD	Infectious bursal disease
IAEA	International Atomic Energy Agency
ICPI	Intracerebral Pathogenicity Index
bELISA	Blocking ELISA

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iELISA	Inhibition ELISA
Ig	Immunoglobulin
LBM	Live bird market
LSI	Laboratoire Service International
mAb	Monoclonal antibody
MDT	Mean death time
MEGA	Molecular Evolutionary Genetic Analysis
MG	*Mycoplasma gallisepticum*
MoA	Minstry of Agriculture
NAHDIC	National Animal Health Diagnostic and Investigation Center
NC	Negative control
ND	Newcastle disease
NDV	Newcastle disease virus
MCMC	Markov Chain Monte Carlo
ML	Maximum-likelihood
NP	Nucleoprotein
NVI	National Veterinary Institute
OD	Optical density
OIE	World Organization for Animal Health
OR	Odds ratio
PASC	Pairwise sequence comparison
PBS	Phosphate buffered saline
PC	Positive control
PI	Percent inhibition
PCI	Posterior credible interval
PM	*Pasteurella multocida*
PPMV	Pigeon Paramyxovirus
PSF	Priority Solidarity Fund
QAP	Quadratic assignment procedure
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
rRT-PCR	Real time RT-PCR
RBC	Red blood cell
RNA	Ribonucleic acid
SARS	Severe Acute Respiratory Syndrome

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Se	Sensitivity
Sp	Specificity
SNNPR	Southern Nations, Nationalities and People's Region
SPF	Specific pathogen free
S/P	Sample-to-positive ratio
UK	United Kingdom
USA	United States of America
VLA	Veterinary Laboratories Agency
VTM	Virus transport medium
WAHID	World Animal Health Information Database

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CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

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1.1 Introduction

Poultry keeping is a common practice in rural households in Ethiopia. The total chicken population in Ethiopia has been recently estimated at 49 million (CSA, 2011). The vast majority of these birds, over 97%, are indigenous chickens kept in an extensive, scavenging system, also known as the backyard or village system, where birds scavenge around the house during the daytime. Three poultry production systems are recognized in Ethiopia, namely the scavenging backyard or village, small-scale commercial and commercial production systems (GRM, 2007; Tadelle Dessie, personal communication) that roughly correspond to sector 4, sector 3 and sector 2, respectively, according to the FAO classification (Demeke, 2007; Alemu *et al*., 2008). In addition, there are several multiplication and rearing centres, which serve as a genetic improvement programme, in the regional states in Ethiopia. Large scale and small-scale commercial poultry production is concentrated in the vicinity of Debre Zeit, near the main market in the capital Addis Ababa, while the local indigenous flocks in the village production system are found all over the country (Figure 1.1). The small-scale commercial system of production is a newly emerging one in urban and peri-urban areas as a household income source, using exotic birds and improved feeding, housing and health-care systems, and produced along commercial lines (GRM, 2007). In the Ethiopian highlands more than 66% of rural households own chickens and the average flock size per rural family was estimated at 7-10 mature chickens (Tadelle and Ogle, 2001; Wilson, 2010).

The latest available figures indicate that village poultry contributes 98.5% of the national egg production and 99.2% of the national poultry meat production (Tadelle *et al*., 2002). This indicates the importance of village poultry production in Ethiopia where chickens serve as a good source of protein and ready cash for villagers. School children often use income generated from selling poultry or eggs sell to buy supplies for school. It is common to see families who invested in small stock such as goats and sheep or other business enterprises from sales of chickens as reported elsewhere (Clarke, 2004; Alders and Pym, 2009). Additionally, village chickens are active in pest control, provide manure which can be used as a fertiliser, are required for special festivals and are essential for many traditional ceremonies (Alders *et al.,* 2010). In Ethiopia honoured guests are served with “doro wot”, a traditional stew made of chicken meat and boiled eggs.

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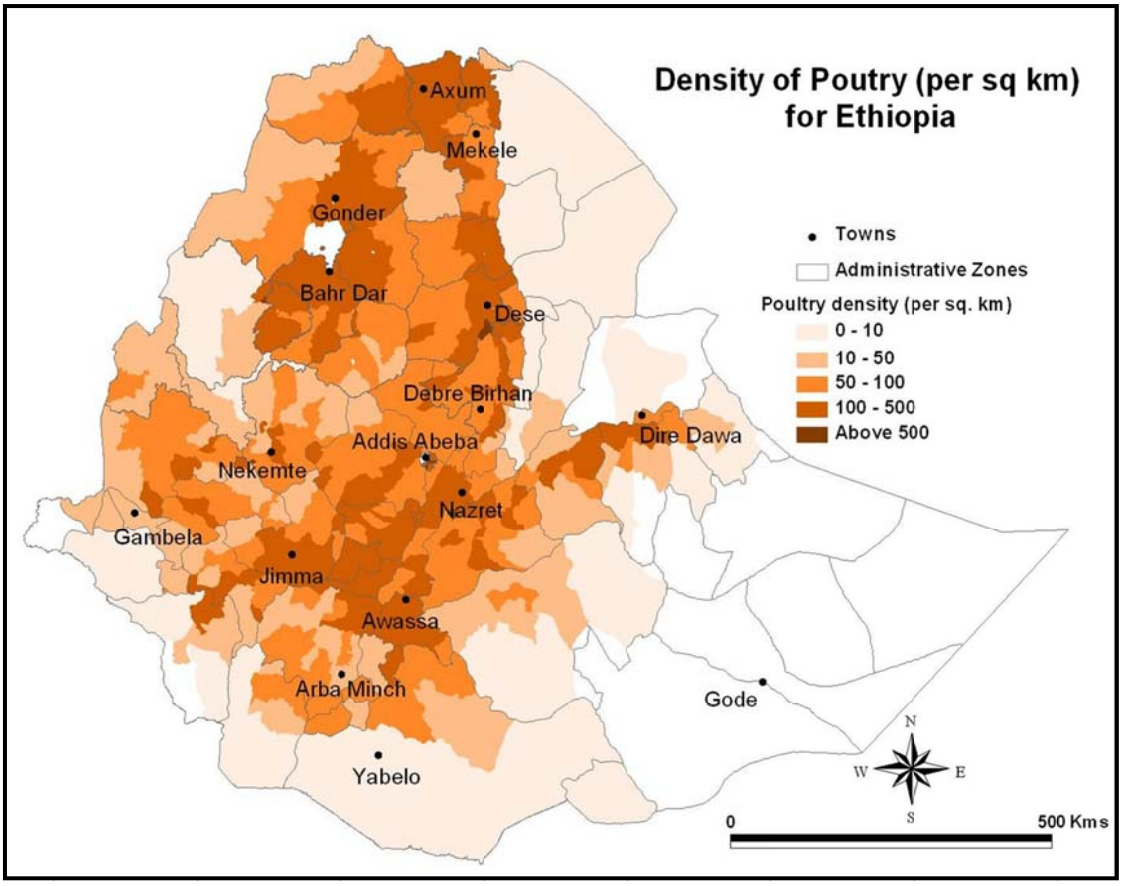

Figure 1.1 Poultry density in Ethiopia

Across the world, especially in developing countries, poultry production is constrained by several factors, of which disease is the most important. Infectious poultry diseases such as Newcastle disease (ND), infectious bursal disease (IBD), mycoplasmosis and pasteurellosis are known to affect village chickens (Bell *et al.*, 1990; Kelly *et al.,* 1994; Chrysostome *et al.*, 1995; Orjaka *et al.*, 1999; Idi *et al.,* 1999; Muhairwa *et al.,* 2001; Ndanyi, 2005; Mushi *et al.,* 2006; Mbuthia *et al.,* 2008). Of these, Newcastle disease is a priority worldwide, and is regarded as the principal factor limiting rural village poultry production (Awan *et al*., 1994; Alders and Spradbrow, 2001). This situation is no different in Ethiopia, but despite this there is neither a control policy nor concerted efforts in disease surveillance, prevention and control. In the commercial sector, regular vaccination programmes are implemented, while in village chickens most farmers are not even aware of the availability of vaccines.

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1.2 Newcastle disease

It is generally accepted that the first reported outbreaks of ND occurred in 1926, in Newcastle-Upon-Tyne, England, whence the name, and in Java, Indonesia (Lancaster, 1976). It is believed that the disease might have occurred earlier elsewhere but had gone unnoticed. In the USA it was initially recognized by mild respiratory and neurological signs which were subsequently termed pneumoencephalitis, and later found to be ND (Ishida *et al.,* 1985). Since then, isolations of NDV have been made from all over the world from both wild and domestic species (Aldous and Alexander, 2001).

Although the exact geographic distribution is difficult to determine because of the widespread use of ND vaccines in commercial poultry, the disease is believed to exist on all the continents, including Africa, and remains a major concern of the agricultural community because of economic losses that have occurred due to illness, death and reduced production or vaccination costs. Newcastle disease is believed to be endemic in the village poultry populations in Africa and is regarded as the most important constraint to the development, survival and productivity of village chicken flocks (Alexander *et al*., 2004; Alders, 2009). Serological and virological evidence has demonstrated the presence of the disease in village poultry in many African countries (Sharma *et al.,* 1986; Bell *et al*., 1990; Bell, 1992; Echeonwu *et al*., 1993; Awan *et al*., 1994; Chrysostome *et al*., 1995; Orjaka *et al*., 1999; Abolnik *et al*., 2004; Zeleke *et al*., 2005b; Otim *et al*., 2007; Servan de Almeida *et al*., 2009; Snoeck *et al*., 2009; Cattoli *et al*., 2010). In countries where ND is endemic, outbreaks of the disease could result in mortalities of upto 100% in a susceptible population depending on the virus strains involved (Spradbrow, 1999; Alexander *et al*., 2004; Alexander, 2011). The virulent form of the disease, with an intracerebral pathogenicity index (ICPI) of ≥0.7 is considered a notifiable disease that must be reported to the World Organization for Animal Health (OIE). In 2004, of the 42 African countries that reported to the OIE, 24 of them reported ND outbreaks, with the incidence ranging from one outbreak (Sudan, Namibia and Niger) to 137 outbreaks (Togo), whilst in four countries the disease was reported to be present only (OIE, 2008a). Between January 2005 and December 2011, several countries reported clinical disease, on average 53 outbreaks (range 29-84) were reported from 54 countries and 3 islands, while in a few countries disease was suspected without confirmation, or information was not available on the disease status at all (WAHID, 2012)

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1.2.1 Aetiology and pathogenicity

Newcastle disease is caused by avian paramyxovirus serotype 1 (APMV-1) viruses, which, with viruses of the other eight APMV serotypes [APMV-2 to APMV-9], have been placed in the genus *Avulavirus*, belonging to the sub-family *Paramyxovirinae*, family *Paramyxoviridae*, (Mayo, 2002; OIE, 2008b). Recently a new serotype of APMV (APMV-10), isolated from penguins, has been confirmed (Miller *et al*., 2010a).

Newcastle disease virus (NDV) is a single-stranded RNA virus with an envelope bearing glycoprotein spikes, of which one function is to initiate haemagglutination (HA). In chickens the pathogenicity of NDV is chiefly determined by the strain of the virus, although dose, route of administration, age of the chicken, health status and environmental conditions could also play a role (Alexander and Senne, 2008). In general, the younger the chicken the more acute the disease is. Newcastle disease virus strains are grouped into five phenotypes based on their pathogenicity and clinical signs induced in infected chickens (Beard and Hanson, 1984, Alexander and Senne, 2008; OIE, 2008b): (i) viscerotropic velogenic, associated with high mortality (up to 100%), often associated with sudden death, without other clinical signs; this type of the virus may cause oedema around the eyes and head; (ii) neurotropic velogenic, marked by sudden onset of upper respiratory signs with high mortality (50-90%, depending on the age of the chicken) and associated with central-nervous signs but diarrhoea is usually absent; (iii) mesogenic with moderate mortality (up to 50%), presenting with respiratory and occasionally nervous signs and also moderate to severe egg production declines for 1-3 weeks; (iv) lentogenic (Hitchner-forms) with mild or sub-clinical respiratory infections; and (v) asymptomatic enteric with sub-clinical intestinal infection and no gross lesions.

Currently NDVs have been classified using two different systems: either genetic lineages (Aldous *et al*., 2003) or classes (Czeglédi *et al*., 2006). The system based on genetic lineage groups NDV in to six lineages and 13 sublineages. Later an additional seven sublineages were suggested (Snoeck *et al*., 2009; Cattoli *et al*., 2010). With class-based system, NDV strains are divided in two clades (class I and class II) (Czeglédi *et al*., 2006). Class I includes almost exclusively low virulent strains recovered from wild waterfowl worldwide. Class II is comprises strains of low and high virulence isolated from poultry and wild birds. From time to time, with the advancement in molecular diagnostic techniques and the broad phylogenetic diversity, new lineages or genotypes are being discovered (Miller *et al*., 2010b) or new

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genotypic classification is being suggested (Diel *et al*., 2012). Molecularly, the different pathotypes are characterized by the amino acid sequence of the cleavage site of the fusion (F) protein (Aldous *et al.*, 2003), recognized as the major determinant in virulence (de Leeuw *et al*., 2005). The presence of multiple basic amino acids, e.g. lysine (K) or arginine (R), located at the C-terminus of the F_1 protein and phenylalanine (F or Phe) at the N-terminus of the F_2 protein, which correspond to the cleavage site of the precursor F_0, is indicative of virulent virus while the low virulent NDV have fewer basic amino acids at this site (OIE, 2008b). Generally in virulent viruses the sequence is ^{113}RQK/RR*F^{117} while viruses of low virulence usually have the sequence ^{113}K/RQG/ER*L^{117}. Accordingly, if the virus is to be virulent for chickens, there appears to be the requirement of a basic amino acid at residue 113, a pair of basic amino acids at 115 and 116 along with a phenylalanine at residue 117 (Alexander, 2009). The protease(s) present in a wide range of host tissues and organs can easily facilitate cleavage of the basic amino acids at these positions in virulent viruses. For low virulence viruses, cleavage can occur only with proteases recognising a single arginine, trypsin-like enzymes, present in areas like the respiratory and intestinal tracts, restricting virus replication in host cells in areas with such enzymes, while virulent viruses can replicate and cause damage in a range of tissues and organs, with a systemic infection (Alexander, 2009).

1.2.2 Diagnosis

Newcastle disease is diagnosed provisionally by its clinical manifestation, and definitively by serology, isolation of the virus and/or molecular assays.

1.2.2.1 Clinical diagnosis

Clinical signs seen in birds infected with NDV vary widely and are dependent on factors such as the virus/pathotype, host species, age of host, concurrent infection with other organisms, environmental stress and immune status (Martin, 1992). Hence, NDV can cause clinical signs ranging from subclinical infections to 100% morbidity and/or mortality, depending on the virulence of the virus and susceptibility of the host. Reviews of the clinical manifestations of infection by different NDV stains have documented variable findings (Cattoli *et al*., 2011). Clinically, ND is diagnosed provisionally by its typical signs such as a state of prostration and depression, ruffled feathers, drooping wings, gasping for air, greenish yellow diarrhoea, and in survivors, the head turned to one side (torticollis), and paralysis of the legs, wings or other neurological signs (OIE, 2008b). Rapid spread of the disease that results in mortality

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over 50% in a naive population can also be considered as a typical characteristic of the disease (Beard and Hanson, 1984). In unvaccinated birds infected with extremely virulent viruses, ND is suspected in any flock in which sudden deaths or high mortality follow severe depression, in appetence, respiratory (open-mouth breathing) or enteric signs and a drastic decline in egg production. Green diarrhoea is frequently seen in birds that do not die early in infection; along with other signs such as, muscular tremors, torticollis, conjunctival swelling, paralysis of legs and wings and opisthotonos (Terregino and Capua, 2009).

However, the lack of pathognomonic clinical signs in many bird species, even for infections with the most virulent viruses, due to their genetic variability and because of similarity with other infections such as highly pathogenic avian influenza (HPAI), poses a serious challenge for the rapid identification and diagnosis of this infection and necessitates the use of other diagnostic techniques.

Other diseases that show one or more clinical signs that are also observed with ND and hence could be considered as differential diagnosis for ND are: fowl cholera, HPAI, laryngotracheitis, fowl pox (diphtheritic form), ornithosis (psittacosis or chlamydiosis) (psittacine birds and pigeons), infectious bronchitis, Pacheco's parrot disease (psittacine birds), IBD (very virulent strains) and salmonellosis (pigeons).

1.2.2.2 Serological diagnosis

Generally speaking, serological assays are carried out to evaluate the immune response following vaccine administration or to detect seroconversion following natural infection. In unvaccinated flocks, excluding newly-hatched chicks with maternally-derived antibodies (MDA), positive serological results could be clear evidence that the birds have been exposed to NDV, but give no indication of the infecting strain. Two techniques are commonly employed to detect antibodies (Abs) in the sera: enzyme-linked immunosorbent assay (ELISA) and haemagglutination inhibition (HI) test. Both techniques have their own merits and limitations.

The HI test is based on the principle that the haemagglutinin on the viral envelope can bring about the agglutination of chicken red blood cells and that this can be inhibited by specific antibodies. The HN is a surface protein believed to be a key in determining the serological classification of viruses currently based on the serological HI assay (Alexander, 1988). The

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HI test is simple to perform, but difficult to standardise amongst laboratories (Beard and Wilkes, 1985). The ELISA works on the principle of recognition of anti-NDV antibodies, of a viral antigen-coated plate, and the bound serum antibodies are consequently detected by anti-chicken antibodies produced in another species that is conjugated in a reporter molecule (Alexander *et al.,* 2004). An ELISA kit for the detection of antibodies against NDV, which is designed to be easily transportable and to give uniform results under widely varying ambient temperatures, has been developed by the Animal Production and Health Section of the Joint FAO/IAEA division (Bell *et al*., 1991). This ELISA was found to have good correlation (r = 0.914) with HI. Tabidi *et al.* (2004), evaluating this ELISA against ND vaccine antibody titre, found that the ELISA can detect higher titres against the vaccine virus and is considered accurate, rapid and sensitive compared to the HI test. Cadman *et al*. (1997) also reported that ELISA detected more antibodies to NDV in naturally exposed and vaccinated ostriches. But HI is considered cheaper than ELISA as no microplate reader is required in addition to the cost of the ELISA kit (Bozorghmehrifard and Mayahi, 2000; Tabidi *et al.*, 2004). However, there have been reports on cross-reactions and cross-protection between the APMV serotypes, especially between APMV-1 and 3 or 7 (Alexander and Senne, 2008; Nayak *et al*., 2012). These, cross-reactions in HI tests may cause some problems that can be resolved by the use of suitable antigen and antiserum controls (OIE, 2008b). However, these tests have limitations due to uncertainty regarding their sensitivity and specificity, as well as, lack of reproducibility under different laboratory conditions (Beard and Wilkes, 1985; Schelling *et al*., 1999; de Wit *et al*., 2007).

As serology cannot give an indication of the infecting strain, the monoclonal antibodies (mAb) technique was introduced to characterize different strains, especially from class II viruses (Collins *et al*., 1998). The development of mAb technology has also resulted in ability to produce highly specific antibodies which enables both the grouping and antigenic differentiation within AMPV-1 (Russel and Alexander, 1983; Jestin *et al.*, 1989). However, since mAbs are directed against single epitopes, individually their ability to detect a broad spectrum of viruses is often limited. In addition, the use of mAb assays for rapid characterization of these viruses is not optimal for class I viruses as most of the mAbs were developed and optimized to recognize class II viruses and fail to recognize viruses of class I (Collins *et al*., 1998; Kim *et al*., 2007b).

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In the past, other techniques for serological diagnosis of ND have been described and used, e.g. complement fixation test, virus neutralization test, agar gel immunodiffusion test, fluorescent antibody test and plaque neutralization, but such methods allow only a generic identification of APMV without any information on pathotype (Cattoli *et al*., 2011).

1.2.2.3 Virus isolation and molecular diagnosis

Whereas serology only gives evidence of exposure, definitive diagnosis of infection is achieved by virus isolation and characterization, most conveniently from live chickens, with samples obtained by swabbing the trachea and the cloaca. The virus can also be isolated from the lungs, brain spleen, liver, and kidneys from recently dead birds or moribund birds that have been killed humanely (OIE, 2008b). Virus isolation is the prescribed test for trade and is considered the 'gold standard' method for validation of other techniques (OIE, 2008b; Terregino and Capua, 2009a). The samples should be put into virus transport medium (VTM), consisting of PBS and antibiotics and/or antifungal agents, to maintain stability of the virus and avoid contamination. Inoculation of the aliquot from swab samples or tissue homogenate into embryonated eggs is done following standard procedures (Alexander, 1998; OIE, 2008b). Generally speaking, more than 85% of ND isolations can be made on the first passage, with less than 10% needing one further passage until the virus adapts itself sufficiently to multiply and cause embryo death (Cattoli *et al.*, 2011). The allantoic fluid is tested for haemagglutination (HA) activity, the key feature for NDV. It can be further confirmed by the HI test, since avian influenza and other paramyxoviruses also cause haemagglutination.

Conventional virus isolation, although considered a sensitive and specific method, has its own limitations, in that it requires eggs from preferably specific pathogen free (SPF) chickens. It takes several days to isolate the virus and undertake the pathogenicity test, and the virus may sometimes not grow successfully because of variation in pathogenicity or the virus may not be available in a sufficient quantity in the specimen or has lost infectivity (Collins *et al.*, 1994; Singh *et al*., 2005; Cattoli *et al*., 2010). With the advent of molecular-based techniques the diagnosis of the disease became easier and more reliable and covered all three aspects of ND diagnosis (detection of virus, characterization, including inference of virulence and epidemiology) quickly, accurately and definitively in a single test (Aldous and Alexander, 2001). The first reverse-transcriptase polymerase chain reaction (RT-PCR) for the

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detection of NDV was introduced by Jestin and Jestin (1991) in infected allantoic fluids employing universal primers to amplify a 238 base pair (bp) section of the F gene. Later on a system that enabled detection of the virus directly in tissue or faeces from infected birds was introduced (Kant *et al*., 1997; Gohm *et al*., 2000). In a study that involved contact between infected and non-infected birds, Gohm *et al*. (2000) showed conjunctiva, lung, caecal tonsil and kidney to be the most suitable organs for NDV detection, which gave most positive results between days 6 and 13 after exposure. Since then several reverse transcription-based molecular techniques including real-time RT-PCR (rRT-PCR), targeting specific portions of the genome have been developed (Wise *et al*., 2004; Fuller *et al*., 2010). Real-time RT-PCR compared to conventional RT-PCR came with the advantage that a post-PCR processing step is avoided, which allows a savings in time (Mackay *et al*., 2002). The molecular techniques afford the possibility of differential diagnosis by multiplex RT-PCRs of pathogens causing similar clinical signs, e.g. avian influenza and NDV (Farkas *et al*., 2007). Although extensive variation among NDVs still poses technical problems, e.g. false negatives because of genetic variability of the nucleotide composition of the region targeted by probes (Cattoli *et al*., 2010), the real and potential advantages of a molecular biological approach to ND diagnosis appear to be overwhelming (Cattoli *et al*., 2011) . In addition to RT-PCR methods, other molecular approaches, such as loop-mediated isothermal amplification (LAMP), have also been developed for detection of NDV (Pham *et al*., 2005).

During an outbreak, it is important to know the pathogenicity of the strain involved. Several in *vivo* methods for pathogenicity assessment have been described (Alexander, 1988a; Terregino and Capua, 2009b). These include clinical signs observed in experimentally infected specific-pathogen free (SPF) chickens, mean death time (MDT) in eggs and plaque formation on chicken embryo, the intravenous pathogenicity index in 6-week-old chickens, and Intracerebral Pathogenicity Indices (ICPI) in day-old chickens. The ICPI is the recommended method for measuring virulence of NDV (OIE, 2008b). These methods are laborious, time consuming and some require animal experimentation (Cattoli *et al*., 2011). Additionally such process delays time if the objective is to put control in place after an introduction of ND. Recently, molecular techniques have allowed testing for virulence based on the presence or not of multiple basic amino acids at the C-terminus of the F_1 protein and phenylalanine (F) at the N-terminus of the F_2 protein, as described above (OIE, 2008b).

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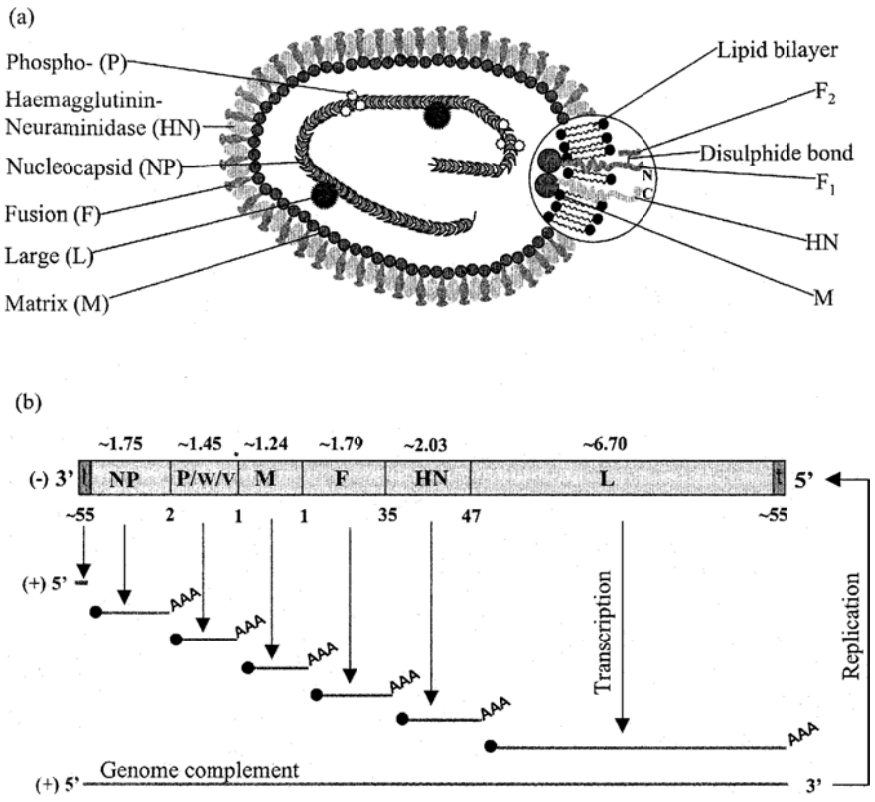

Figure 1.2 The structure of Newcastle disease virus (from Yusoff and Tan, 2001).

1.2.2.4 Nucleotide sequencing and phylogenetic studies

Identification of the virus as NDV and an estimate of its pathogenicity provide no information that would enable assessment of the source of the virus and its spread (Aldous and Alexander, 2001). In recent years, developments in the enzymes and equipment available for nucleotide sequencing have transformed it from a laborious and complex technique to one that is almost fully automated. RT-PCR offers the possibility of subsequent sequencing of the amplified DNA allowing pathotyping of the isolate (Seal *et al.*, 1995; Gohm *et al.*, 2000). Additionally, development of improved techniques for nucleotide sequencing, the availability of sequence data of more NDVs in databases, and the demonstration that even relatively short sequence lengths could give meaningful results in phylogenetic analyses have led to a marked increase in phylogenetic studies (Aldous and Alexander, 2001). The NDV genome is composed of six genes encoding their corresponding structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), and RNA polymerase (L) (Figure 1.2). The HN and F are glycoproteins that allow binding and fusion of the virus to the host cells to initiate a NDV infection (Miller *et al.*, 2007). Phylogenetic

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studies of both the F protein and the HN protein genes of NDV have been used for molecular epidemiologic analysis and characterization (Ballagi-Pordany *et al.,* 1996; Ke *et al*., 2001; Otim *et al*., 2004) and to group NDV into specific lineages or genotypes by estimating the average evolutionary divergence over sequence pairs (Westbury, 2001; Tamura *et al*., 2004). Following phylogenetic analysis of the aligned sequences, it is possible to group the viruses for epidemiological studies (temporal and spatial distribution and spread) and reliably predict the pathotype and genotype of each virus.

1.2.3 Epidemiology of Newcastle disease

1.2.3.1 Host range

The vast majority of birds are susceptible to infection with NDV of both high and low virulence; although the clinical disease may vary greatly from one species to another, depending on factors such as the virus strains, host species, age of the host, environmental stress and immune status (OIE, 2008b). Kaleta and Baldauf (1988) reported that, in addition to domestic avian species, at least 236 bird species have been reported to be susceptible to natural or artificial NDV infection. Chickens are highly susceptible, but other poultry species can be infected with NDV, and may play a role in the spread of NDV in extensively managed poultry. These birds include ducks, geese, turkeys, doves and guinea fowl. Such birds can become infected with NDV, shed the virus, and act as a source of infection for chickens, even if they do not develop clinical signs (Martin, 1992; Alexander *et al*., 2004; Otim *et al*., 2006).

1.2.3.2 Transmission and spread

The major mode of transmission appears to be by the faecal-oral route in free range scavenging poultry, with the respiratory route playing a role where there are close bird-to-bird contacts (Martin, 1992). For the enteric form of the viruses, including avirulent and pigeon variants, the faecal-oral route appears to be main method for transmission from bird to bird (Alexander *et al*., 1984). Virus is shed during incubation, during clinical stage and for a varying but limited period during convalescence (Kahn and Line, 2005). In experimentally infected chickens Gohm et al. (2000) have managed to detet virus genome in faces and conjunctiva 1 day post infection (dpi) in RT-PCR where faecal samples showed positive

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results until the end of the experiment (28 days). Similarly Westbury *et al*. (1984) have reported excretion of virulent virus from challenged birds up until 14 days. In experimental infection of NDV in pigeons, de Oliveira Torres Carrasco *et al*. (2008) has also established that viral genome shedding was observed between 11 to 13 dpi and had occurred for up to 20 dpi. Excretion of virus is dependent on the organs in which the virus multiplies, which may vary with viral pathotype (Alexander, 2009). The issue of vertical transmission (passing of virus from mother to progeny via the embryo) has not yet been resolved. However, Roy and Vanugopalan (2005) obtained NDV isolates, some of which were velogenic, from the gut contents of day old chicks in commercial hatcheries, and concluded that the possible source was vertical transmission from breeder hens. Earlier Pospisili *et al.* (1991) isolated lentogenic virus from gut contents of non-vaccinated chicks from hatching up to day 25. In addition, Capua *et al*. (1993) described an unexpected isolation of virulent virus from chicken embryos as well as from hatching chicks. Chen and Ching-Ho Wang (2002) using both clinical and experimental evidence proved that embryos infected *in ovo* with a low titre of NDV can hatch and contain NDV after hatching, suggesting that NDV can spread through eggs due to contamination during laying if the parent stock is infected.

Once in the poultry flock, the main means by which virus can spread from area to area, as summarized by Alexander *et al.* (2004), are movement of live birds, movement of people and equipment, movement of poultry products and contaminated poultry feed or water. Infected chickens are considered as the usual sources of infection in villages and spread is usually attributed to movement of chickens through live bird markets (Martin, 1992; Spradbrow, 1999). The close contact of chickens from different areas at local markets followed by movement back or transportation to various localities from the market can undoubtedly facilitate the rapid spread and persistence of the disease among village chickens. Airborne spread of the disease has also been considered important in commercial flocks (Hugh-Jones *et al.*, 1973) and this has been substantiated under experimental conditions (Li *et al*., 2009). However, the success of this route of transmission will depend on many environmental factors, such as temperature, humidity and stocking density.

The role of wild birds in the spread of NDV is not fully understood (Martin, 1992). However, for village poultry, they may serve as source of NDV, as the chance of contact with wild birds is higher than for intensive poultry (Kahn and Line, 2005) and several species of birds may be infected with NDV with or without clinical signs (Kaleta and Baldauf, 1988). NDV

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has been diagnosed in wild pigeons or other wild bird in several occasions (Echenwu *et al*., 1993; Kuiken *et al*., 1998; Alexander, 2000); these can easily access rural chickens or indirectly contaminate feed or drinking water. Newcastle disease outbreaks were also reported in commercial poultry farms in Great Britain that was caused by genetically similar ND viruses in Scandinavian countries and attributed to wild bird migration (Alexander *et al*., 2004). In an attempt to detect NDV haemagglutinating activity in semi domestic free-range birds (*Numida meleagris* and *Columba livia domestica*), Wambura (2010) detected antibodies to NDV in serum samples collected from guinea fowl and pigeons, indicating the risk of transmission to village chickens. In experimental infection of domestic chickens with pigeon origin NDV (PPMV), after passage in chickens, increased virulence of the passaged PPMV-1 isolates was demonstrated (Kommers *et al*., 2001) and spread from feral pigeon population into domestic chickens caused several outbreaks among commercial chickens in Great Britain (Alexander *et al*., 1984).

1.2.3.3 Persistence of the virus

In the outside environment survival times are dependent on temperatures and relative humidity. In the poultry premises, NDV has been reported to survive on feathers for 255 days (Leighton and Heckert, 2007) and in litter for 42 to 53 days (AUSVETPLAN, 2010). It was also established that the NDV can survive in poultry faeces for several days to weeks depending on temperatures (Kinde *et al*., 2004). NDV has the potential to persist in the meat and other products obtained from infected birds. Newcastle disease virus was isolated from the carcasses of frozen poultry for over 730 days and from buried carcasses for 121 days (Hess, E cited in Pirtle and Beran, 1991). Virus can survive also in poultry offal. Bone marrow and muscles from slaughtered chickens are the ideal places for the virus to survive.

Chakrabarti *et al*. (2008) have demonstrated that flies exposed to either infectious food source for 24 hours became transiently infected with virus and could carry infective dose for a susceptible chicken for 1-4 days depending on the source of contamination. Capua *et al*. (1993) isolated virulent NDV from fertile eggs and live progeny of vaccinated breeders. Virulent ND virus has been detected in infected vaccinated flocks for more than four months (Krauss 1965; Utterbuck and Schwartz, 1973). Virus can remain latent in the trachea and has been recovered by organ culture from the trachea of one bird 120 days after infection (AUSVETPLAN, 2010). The infectivity of the virus can be destroyed by range of

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environmental and chemical factors such as heat, irradiation and pH, although this varies depending on virus strains, the duration of exposure, viral quantity and medium of suspension (Alexander and Senne, 2008)

1.2.3.4 Prevention and control of Newcastle disease

Many countries, particularly in the developed world, have their own prevention and control polices, which mainly focus on vaccination, quarantine and/or implementation of biosecurity measures like movement controls for anything that could have become contaminated with virus including importation of specimens (AUSVETPLAN, 2010; Alexander, 2011).

1.2.3.4.1 Vaccination

The most widely accepted preventive strategy for ND worldwide is vaccination of birds. The basis for immunity to NDV includes circulating antibodies, secreted antibodies producing mucosal immunity, and cell mediated immunity (Alexander and Senne, 2008). Chicks born of vaccinated and/or recovered hens from natural infection acquire passive immunity via the yolk. Passive immunity provided by maternal antibodies was first demonstrated in 1893 by the transfer of immunity to tetanus toxin from vaccinated hens to chicks (Klemperer, 1893). Since then maternally-derived antibody has become an important factor in deciding vaccination policy in the poultry industry.

Both inactivated and live vaccines have been developed and are in use worldwide under different trade names. Live vaccines are usually produced from mild viruses, i.e. lentogenic or avirulent strains such as Hitchner-B1, LaSota/46, F and V4, or mesogenic strains such as Roakin, Mukteswar and Komarov, which have proven efficacy in prevention of ND or in controlling ND outbreaks in many parts of the world. The use of mesogenic strain vaccines is limited in countries where the disease is not endemic (OIE, 2008b). Live vaccines have the added advantages of quick stimulation of local immunity and the ability to protect soon after vaccination, as well as ease of application through mass medication via drinking water. The immune response induced by a live vaccine is influenced by the type of viruses, the degree of attenuation, the delivery route, the site of replication as well as the age and immune status of the bird (Schijns *et al*., 2008). The vaccine virus also spreads easily from successfully vaccinated birds to those that were not. However, it may produce disease in vaccinated birds

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depending on environmental conditions and the presence of other complicating infections (Alexander and Senne, 2008).

Vaccination in intensive systems

Intensive poultry production systems are characterized by the presence of high numbers of poultry kept in one place, single age groups and confined housing. Newcastle disease control relies on regular vaccination of chickens using live vaccines delivered by the intra-nasal, intra-ocular or intra-muscular/subcutaneous routes, by aerosol spray or in drinking water. In such systems vaccine dose and ambient temperature (easy to control) are not a problem, and a variety of vaccines have been used, including LaSota (live vaccine, thermolabile); Hitchner-B1 (live vaccine, thermolabile), and I-2 (live vaccine, thermotolerant).

Vaccination in village production systems

ND control in scavenging backyard chickens remains a challenge in Africa and elsewhere in the developing world, because of small flock sizes and free roaming systems that pose difficulties in catching and vaccinating. Additional constraints that hinders effectiveness of conventional control strategies in village chickens includes the technical and/or logistical demands, *i.e*. lack of heat stable vaccines, vaccines produced in large dose units and need for skilled vaccinator (Spradbrow, 1999). Some of the logistic problems may be less important if a thermotolerant NDV strain is used that does not depend on a cold chain. The development of the thermotolerant V4 and I-2 vaccine virus by the Australian Centre for International Agricultural Research (ACIAR) was significant step in overcoming the limitations associated with conventional vaccination. It provided more heat stable variants, as well as the prospects of feed-based vaccine delivery (Spradbrow, 1992). Trials with V4 and I-2 strain vaccines have shown that it was possible to deliver the vaccine virus to chickens in a feed-carrier, and this has been attempted in several African countries, including Ethiopia (Nasser *et al.,* 2000). An extensive review on the use of feed-carrier based vaccine was published by Oakeley (2000). However, no single grain has been identified as an ideal food-carrier for large-scale use. Nevertheless, successful cases of ND vaccination of village chickens, using thermostable vaccine, have been documented in ACIAR research results (Foster *et al.*, 1999; Spradbrow, 2005). The AusAID Southern Africa Newcastle disease control project has documented a successful model for control of ND, using thermotolerant I-2 vaccine, in village chickens by introducing an effective and sustainable system through community participation (AusVet,

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2006). Lessons learned over fifteen years in ND control in village chickens has been documented by Alders *et al*. (2010). In most developing countries the resources to implement vaccination campaigns on the scale and frequency necessary to cover their scavenging backyard poultry flocks are limited (Sonaiya *et al*., 1999), and sometimes less attention is paid to the needs of village chicken owners.

Newcastle disease vaccination in Ethiopia

In Ethiopia formal vaccination in commercial farms was started in 1974 using the Hitchner-B1 produced at the National Veterinary Institute (NVI) (Bawke *et al*., 1991). However, there is as yet no formal control strategy for village chickens in Ethiopia. ND vaccination is mostly practiced in commercial flocks. Currently different types of ND vaccines (Hitchner-B1, LaSota/46, and thermotolerant I-2) are produced locally by the NVI and provided on request to the regional agricultural bureaus and commercial and small-scale commercial producers. Although small in scale the poultry development package, based on the distribution of exotic breed chickens, being implemented by the Ministry of Agriculture (MoA) exercises vaccination of chickens intended for distribution to farmers. However, the practice is limited to poultry multiplication and distribution centres. In general, there is no clear national policy to vaccinate village chickens in Ethiopia, although a village-based poultry development programme, targeting indigenous, improved and exotic breeds separately, has recently been developed (Tedese Sori, 2009, personal communication). Individual rural producers in Ethiopia do not have much awareness about the presence of vaccination for chickens in most cases and they sometimes resort to using ethno-veterinary and magico-religious practices (Ashenafi, 2000; Tadelle and Ogle, 2001) although their effectiveness has not been scientifically tested. Even if they have heard about the presence of vaccine they do not know how to acquire it.

1.2.3.4.2 Biosecurity and quarantine measures

The other option to prevent disease is through application of bio-security measures and imposition of effective quarantine measures. To this end countries in the developed world may have their own rules and regulations or preventive strategies. The preventive measure here mainly focuses on sanitary prophylaxis, i.e. proper biosecurity measures to be applied in poultry premises. In village chickens the biosecurity is generally considered very poor, making disease prevention and control difficult (Pagani and Wossene, 2008).

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In the developed world, within a country, quarantine measures may be imposed depending on the level of perceived risks whenever there is doubt about the infections, to limit the spread of disease by prohibiting the movement of birds, products and materials to or from infected/suspected farms, including movement of humans (AUSVETPLAN, 2010). For safe trade purposes, the OIE terrestrial animal health code recommends the times for industry standard temperatures suitable for the inactivation of ND virus in meat should be (at least) at 65°C for 4 minutes, while for live animal importation the veterinary authority should require the presentation of an international health certificate with different requirements depending on the disease status of exporting countries (OIE, 2009).

1.2.4 Newcastle disease in public health and bioterrorism

Newcastle disease virus is a human pathogen of minor importance. Human disease caused by ND virus has been reviewed by Khan (1994). The common result of ND virus infection in humans is conjunctivitis, which may be severe but is of only a few days duration and without residual effect once resolved, unless complicated by secondary pathogens. This was recently seen in a veterinary student sampling sick chickens with ND at the NAHDIC premises, presumably infected via respiratory droplets (personal observation). Newcastle disease was also one of more than a dozen agents the United States researched as potential biological weapons before the nation suspended its biological weapons program (http://articles.janes.com/articles/Janes-Nuclear,-Biological-and-Chemical-Defence/Newcastle-disease-virus.html). Interestingly, NDV may have role in a new therapeutic approach using viruses for the treatment of cancer, termed oncolytic virotherapy, since it has an inherent oncolytic property that has shown benefits to cancer patients (Ravindra *et al*., 2009; Schirrmacher and Fournier, 2009). Its antineoplastic efficacy appears to be associated with three properties of the virus: selective replication in tumour cells, the capacity of some strains of NDV to lyses tumour cells and immunostimulatory capacity (Fournier *et al.,* 2012).

1.2.5 Newcastle disease and other poultry diseases in Ethiopia

In Ethiopia ND is considered to be the most important poultry disease. The first documented outbreaks of ND in Ethiopia date back to 1971; the report was from a small poultry farm in Asmara, the capital of the present Eritrea, then part of Ethiopia (NVI, 1974). However, it is possible that the disease occurred before this time in poultry populations in Ethiopia. From that time onwards the disease spread widely and is recognized as endemic in Ethiopia,

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including commercial farms (Bawke *et al*., 1991; Nasser, 1998). The disease is well known by farmers in Ethiopia, especially in its virulent forms, and locally known most commonly as '*fengel*', which means sudden dorsal prostration that signifies the acuteness and severity of the disease. Different local names are employed in different parts of the country. Although there is no detailed study documenting mortality due to ND in Ethiopia, Nasser (1998) has reported 50% mortality in local chickens around Addis Ababa, Debre-Zeit and Nazreth. Halima *et al.* (2007), in their study of village chickens in North-west Ethiopia, identified seasonal outbreaks of ND as the major cause of death.

Published information on the epidemiological and ecological features of ND outbreaks in Ethiopia is scanty. Reports by the veterinary department to the OIE recorded on average 50 outbreaks per year during 2005-2011 (WAHID, 2012). This likely greatly underestimates the true figure as the majority of the outbreaks are believed not to be reported by farmers, who assume that there is no benefit in doing so. A few cross-sectional seroprevalence surveys, based on the HI test, conducted in village chickens in different parts of Ethiopia have suggested that the prevalence was variable. Zeleke *et al*. (2005b) in the study conducted in the wet southern and drier Rift Valley districts reported a prevalence ranging from 14-23 % while no positive sera were detected in the wet mountainous areas investigated. Tadesse, *et al*. (2005) analysed 180 chicken sera from three agro-climatic areas for NDV antibodies, yielding a seroprevalence ranging from 29-38%. Regasa *et al*. (2007) reported a seroprevalence of 12% in Shashamene and 10% in Adami Tulu districts. Getachew (2009) documented an overall seroprevalence of 5.6% (range: 3.2-8%) using commercial ELISA in South West Shewa zone.

The clinical signs of ND that causes considerable losses in the poultry sector are directly related to the pathogenicity of the virus strains involved. While the velogenic strains cause severe clinical disease and higher mortality, disease caused by mesogenic and lentogenic strains are relatively mild. A few ND outbreaks investigated in Ethiopia between 1984 and 1997, were all attributed to velogenic virus strains (Nasser, 1998), however no information on the characteristics of those viruses is available. Earlier isolates characterized by MDT and ICPI from a chicken farm at Alemaya were identified as velogenic viruses (Lefevre and Martel 1975). Recently, from a few chicken samples sent to the Veterinary Laboratories Agency (VLA) at Weybridge, UK, both APMV-1 and PPMV-1 were isolated indicating also the circulation of pigeon variant virus in domestic chickens.

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Apart from ND, several other infectious diseases of poultry have also been diagnosed in Ethiopia and implicated in constraining poultry production, mainly in the intensive production systems. Concurrent infection of several poultry diseases, including mycoplasmosis, salmonellosis, IBD and colibacillosis, has been reported from broiler farms in Ethiopia (Chanie *et al*., 2009). Zeleke *et al.* (2005a) reported a mortality rate of about 50% due to IBD in 20-45 day old chickens in a layer and broiler farm while it was also diagnosed from a government-owned poultry multiplication centre (Woldemariam and Wossene, 2007). IBD in village chickens has been serologically documented from the northwest and central part of Ethiopia (Mazengia *et al*., 2009; Degefu *et al.,* 2010). The host-specific and transovarially transmitted salmonellae, *S. pullorum* and *S. gallinarum* were also isolated from sick and moribund meat and egg type chickens (Lobago *et al*., 2003). The presence of *Mycoplasma gallisepticum* (MG), resulting in chronic respiratory disease, has also been reported in commercial farms in Ethiopia (Alamargot, 1987; Cardinale, 2005; Chanie *et al*., 2009). In contrast their occurrence in village poultry production systems has rarely been documented while they could be a potential reservoir of these and several other pathogens which may jeopardize the development of small-scale commercial poultry production in Ethiopia.

1.3. Study rationale and research questions

ND is believed to be the major limiting factor for village chicken production, but it is likely that several other infectious poultry diseases are present and may play a role in affecting backyard poultry production in Ethiopia. However, the status of these diseases in scavenging backyard poultry in Ethiopia is largely unknown. The key to increasing profitability of backyard poultry production is to know which diseases are prevalent in an area (Bell, 2009).

Although ND is endemic in Ethiopia since its first occurrence, the epidemiology of the disease at household level is poorly understood or not adequately addressed. Published information on the epidemiological features of ND at individual and household levels are scanty in Ethiopia. Although a few individual serological reports were documented no published document or report was found on the virological status of village chickens.

Similarly, factors that facilitate introduction and spread of the disease at the household flock level have not been investigated in Ethiopia. Understanding of specific risk factors is a requirement for formulating an effective control programme or any other measure envisaged.

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Some of the risk factors implicated elsewhere for the rapid spread and persistence of infection among village chickens have been the presence of latently infected carrier chickens, village poultry dynamics (selling, buying, giving), absence of preventive measures, unrestricted contact between village flocks and other poultry species including wild birds, disposal methods for poultry waste and carcasses, and re-stocking practices (Awan *et al*., 1994; Spradbrow, 1999; Otim *et al.,* 2007; Njagi *et al*., 2010; Rasamoelina Andriamanivo *et al*., 2012). Detail review of different risk factors in village chickens was provided by Miguel *et al*. (2012). There is no study to date that has attempted to identify the risk factors that determine the occurrence of the disease or exposure to NDV in household chickens in Ethiopia and no research has been published in this regard.

Several virus strains are considered to circulate between markets and villages in Ethiopia, where virulent strains are believed to dominate on the basis of the clinical signs of ND that cause considerable losses in the poultry sector which are mostly related to the pathogenicity of the virus strains involved. Sequence analysis, pathotyping and detailed genetic characterization of NDV strains detected in rural poultry are of paramount importance to understand the circulating genetic lineage characteristics in a particular geographical area and trace their epidemiological relationships (Seal *et al*., 1995; Gohm *et al*., 2000; Singh *et al*., 2005). However, the genetic characteristics of the virus strain(s) circulating in Ethiopia are largely unknown and no detailed characterization was reported or is available in the literature.

Serological tests such as ELISA and HI are often relied upon for the diagnosis and surveillance of ND, indirectly through the detection of antibodies produced following vaccination or infection by NDV. Commercial ELISAs, that are validated mostly in SPF flocks or experimental situations, and are widely used for the evaluation of immune status in commercial flocks, are also being used for the diagnosis and surveillance of NDV antibodies in village chickens. However, these tests have limitations due to uncertainty regarding their sensitivity and specificity as described above.

Furthermore, ND can spread by contact between birds, which is facilitated by the practice of taking birds to markets from an infected flock, then from market to market, or taking unsold or purchased birds back from market to villages. Understanding such contact networks, between markets, would help to investigate potential disease spread pathways and evaluates the role of markets in the epidemiology of ND. However, our knowledge of how poultry

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markets in Ethiopia are connected to each other by the movement of people, including quantitative description of movements of chickens and traders, is limited.

1.4 Study setting

The rapid and wide geographic spread of HPAI H5N1 in recent years has drawn attention to the neglect of village poultry health in developing countries, including Ethiopia. This crisis has presented an opportunity to invest in surveillance and research. The study described in this thesis was funded by the Priority Solidarity Fund (PSF) of the French Ministry of Foreign and European Affairs, under the "Ecology and Epidemiology of Avian Influenza in Southern Countries" GRIPAVI project and was part of a more global approach aimed at modelling avian influenza (AI) and ND dissemination among wild birds and domestic poultry populations.

The general objective of the project was to improve understanding of ecological and epidemiological processes in order to develop recommendations concerning risk evaluation and management towards decision-making. The project was concerned with two diseases, AI and ND, and was particularly focused on Africa. The specific objectives were to improve knowledge of AI and ND epidemiology and ecology in tropical zones, to convert this knowledge into control and monitoring tools and to train and inform role players.

Field studies were implemented in six countries (Ethiopia, Madagascar, Mali, Mauritania, Vietnam, and Zimbabwe) and were based on "observatories" in each of these countries dedicated to a set of thematic areas according to each observatory's own characteristics. The complementarities of protocols aimed to provide a "meta-observatory" enabling generic recommendations in terms of risk analysis and control measures to be made.

With regard to previous collaboration existing between Ethiopia and the *Centre de Coopération Internationale en Recherche Agronomique pour le Développemen*t (CIRAD), and the situation of the country in view of highly pathogenic poultry diseases such as ND, Ethiopia was included as a partner and a PhD programme on the epidemiology of ND was funded.

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1.5 Description of the study area

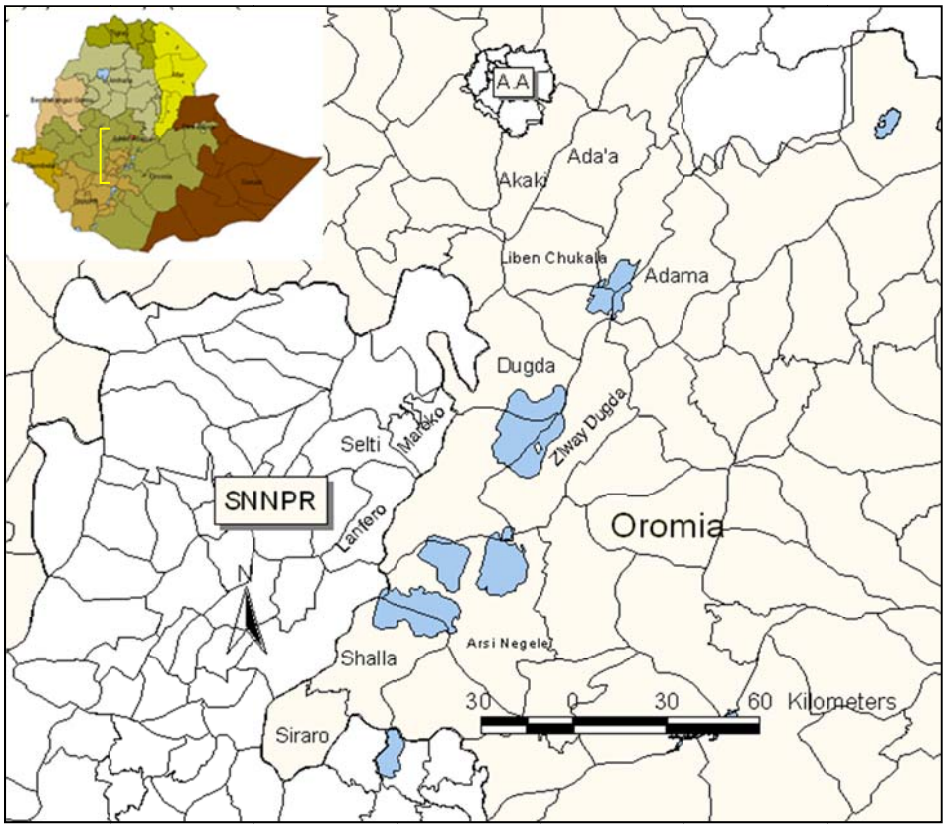

Figure 1.3 Map of Ethiopia showing the study area

Ethiopia is divided into nine administrative regions and then into zones and woredas (districts). The study was conducted mainly in the mid-Rift Valley (part of the Great Rift Valley of eastern Africa) that included the East Shewa and West Arsi zones of the Oromia administrative region. Depending on the nature of the different study components, the area was extended to other neighbouring zones and woredas of the Oromia administrative region and Southern Nations, Nationalities and People's Region (SNNPR). The study area mainly covered the mid-Rift Valley area at an altitude of 1500-1900 m above sea level, where many water bodies are present (Figure 1.3). The water bodies in this area are known to be the resting ground for migratory birds during the European winter. It roughly lies between 07°12' to 08°52' North and 38°21' to 39°33' East. The climate of the area is sub-tropical and humid with a mean annual rainfall of 750-1100 mm, increasing towards highland escarpments, and average minimum and maximum temperatures of 8 and 35°C, respectively. Some areas have a semi-arid climate with an erratic and low rainfall, averaging between 500 and 900 mm per

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annum (Zonal Agricultural office). Broadly, there are two main seasons recognized in the study area: the wet season from June to September, sometimes extending to October, and the dry season from October to May (NMA, 2009). The type of agriculture in the study area is small-scale mixed crop and livestock farming.

The area was specifically selected for its relatively high poultry and human population density in the region (CSA, 2007) and the presence of wetlands where other complementary studies were also undertaken. The area has also numerous rural and urban live bird markets. Further details on description of the study area and the selection of epidemiological units are provided under each chapter.

1.6 Objectives of the study and outline of the thesis

The first objective of this study was to assess the seroprevalence of ND and other infectious poultry diseases in village chickens at live bird markets. Chapter 2 describes the status of ND along with the other established poultry diseases based on repeated serological surveys in live bird markets.

Secondly, the study was designed to assess the NDV exposure and virus circulation at household flock level in villages and associated markets. Chapter 3 describes the exposure of household flocks to NDV in a cross-sectional study using a multistage random sampling design with repeated sampling periods that involved both serology and molecular detections.

A further objective of the study was to understand risk factors associated with ND seropositivity and incidence NDV exposure at household flock level. Chapter 4 describes risk factors associated with NDV seropositivity and incidence of NDV exposure assessed in household flocks based on information generated from a structured questionnaire survey coupled with serological assessments.

In addition, the study was designed to isolate and molecularly characterize the NDVs circulating in the study area and investigate epidemiological relationships of the isolates from chickens in village and markets. Chapter 5 describes the molecular characterization and phylogenetic analyses, on virus isolates obtained from chickens at live bird markets and villages, based on rRT-PCR and complete F and HN gene sequencing

Furthermore, the study aimed to evaluate test parameters of different serological techniques

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available for the diagnosis of NDV antibodies that could be applied under field conditions for different purposes. Chapter 6 describes the results of Bayesian analysis used to evaluate the performance of two commercial ELISA kits (a blocking and an indirect ELISA) and HI test, in the absence of a gold standard, for their ability to detect antibodies to NDV in chicken serum from villages and live bird markets

Finally, the study was planned to describe patterns of live poultry trade, spatially and temporally, to understand the contact network of poultry markets in the selected study area during two different periods of a year, as well as to evaluate their potential for influencing disease spread and inform surveillance approaches applicable for infectious poultry disease at markets where live birds are sold. Chapter 7 describes the market trade movement patterns for live chickens established using social network analysis, for two different periods of the year 2010, that represented the high and low movement seasons for chickens trade, and document surveillance approaches to follow in these markets.

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CHAPTER TWO

SEROPREVALENCE OF NEWCASTLE DISEASE AND OTHER INFECTIOUS DISEASES IN BACKYARD CHICKENS AT MARKETS IN EASTERN SHEWA ZONE, ETHIOPIA

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2.1 Abstract

This study was conducted to estimate the seroprevalence of Newcastle disease (ND), *Pasteurella multocida* (PM) infection, *Mycoplasma gallisepticum* (MG) infection and infectious bursal disease (IBD), and to assess the level of concurrent seropositivity during the dry and wet seasons of the year 2010. A total of 234 and 216 sera were collected during the dry and wet seasons respectively from unvaccinated local breed backyard chickens at four live poultry markets in two woredas (districts) of Eastern Shewa zone, Ethiopia, and were tested using commercial enzyme-linked immunosorbent assay (ELISA) kits. The overall seroprevalence of ND, PM, MG and IBD was 5.9%, 66.2%, 57.7% and 91.9% respectively during the dry season, and 6.0%, 63.4%, 78.7% and 96.3% respectively during the wet season. The seroprevalence of MG was significantly higher ($P<0.001$) during the wet season than during the dry season and significantly higher ($P = 0.002$) in Adami-Tulu-Jido-Kombolcha (ATJK) woreda (74%) than in Ada'a woreda (60%). Area and season had no significant effect on the seroprevalence of ND, IBD and PM, indicating the widespread presence of those pathogens throughout the year in the study area. Of all the chickens tested, 85.6% had antibodies concurrently to more than one of the pathogens investigated. Out of the four diseases evaluated, birds were concurrently seropositive to more diseases during the wet season (median = 3) than during the dry season (median = 2) ($P = 0.002$). As serology is not able to distinguish between strains, further studies are warranted to better understand the circulating strains, their interactions and their economic effect on backyard poultry production in Ethiopia.

Key words: Newcastle disease, infectious disease, backyard chickens, Ethiopia

2.2 Introduction

Like elsewhere in the developing world, backyard poultry rearing is a common practice in rural Ethiopia. Village based backyard poultry, characterized by traditional production methods and local breeds, represents 98% of the total Ethiopian poultry population of 49 million (CSA, 2011). The sector provides eggs and poultry meat to most rural and many urban consumers (Tadelle, 1996). However, the productivity of backyard chickens is hampered by several factors, including a variety of infectious diseases. In Ethiopia an

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estimated 40-60% of newly hatched chicks die before reaching maturity, mainly due to disease and predation (Tadelle *et al.,* 2003a). Farmers in Eastern Shewa zone tend to consider any disease that causes moderate to high mortality to be Newcastle disease (ND), known locally as *Fengil or Fenqil,* as also reported elsewhere (Sonaiya and Swan, 2004). It is therefore likely that many other infectious diseases have, for a long time, been described incorrectly as ND. Mortalities of backyard chickens may also result from other viral or bacterial diseases, either individually or concurrently. Several viral and bacterial infectious diseases, including ND, *Mycoplasma gallisepticum* (MG) infection, infectious bursal disease (IBD) and Marek's disease have been described in commercial poultry farms in Ethiopia (Alamargot, 1987; Nasser *et al.*, 2000; Lobago and Woldemeskel, 2004; Zeleke *et al*., 2005a, b; Chanie *et al.,* 2009), but their occurrence in scavenging backyard poultry production systems has rarely been documented. The key to increasing profitability of backyard poultry production is to know which diseases are prevalent in an area (Bell, 2009). Workers in other African countries have documented the presence of many viral and bacterial diseases in backyard production systems (Bell *et al.*, 1990; Kelly *et al.,* 1994; Chrysostome *et al.*, 1995; Orjaka *et al.*, 1999; Idi *et al.,* 1999; Muhairwa *et al.,* 2001; Ndanyi, 2005; Mushi *et al.,* 2006; Mbuthia *et al.,* 2008). It is therefore also likely that infectious diseases such as ND, IBD, mycoplasmosis, and pasteurellosis could play a role, individually or concurrently, in scavenging backyard poultry health in Ethiopia. In addition, backyard poultry could be a potential reservoir of these pathogens which could jeopardize the development of semi-commercial poultry production in Ethiopia.

Newcastle disease is caused by avian paramyxovirus serotype 1 (APMV-1), which, with viruses of the other nine APMV serotypes (APMV-2 to APMV-10), have been placed in the genus *Avulavirus*, belonging to the sub-family *Paramyxovirinae*, family *Paramyxoviridae* (OIE, 2008b; Miller *et al*., 2010a). It has a worldwide distribution and is regarded as one of the most important constraints to the development of backyard poultry production (Alexander *et al.*, 2004; OIE, 2008b). Serological and virological evidence has shown the presence of the disease in backyard poultry in many African countries (Bell *et al.*, 1990; Echeonwu *et al.*, 1993; Alders *et al.,* 1994; Chrysostome *et al.*, 1995; Orjaka *et al.*, 1999; Snoeck *et al.*, 2009; Servan de Almeida *et al.,* 2009), and the disease has long been known to be endemic in Ethiopia (NVI, 1974). Limited previous serological surveys of ND based on haemagglutination inhibition (HI) titre antibody detection in backyard chickens in Ethiopia

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have found that 11-38% of chickens had detectable antibodies (Tadesse *et al.*, 2005; Zeleke *et al.*, 2005b; Regasa *et al.*, 2007).

Infectious bursal disease (IBD) is a highly contagious, immunosuppressive infection of immature chickens, caused by IBD virus that belongs to genus *Avibirnavirus* of family *Birnaviridae*, with a worldwide distribution (Sharma *et al.,* 2000; van den Berg *et al*., 2004). Two serotypes of IBD virus strains are described: 1 and 2. Serotype 2 strains classified as apathogenic and serotype 1 strain, pathogenic to chickens, classified into several pathotypes, from mild to hypervirulent, according to their virulence (van den Berg *et al*., 2004). IBD virus antibodies amongst backyard chickens have been reported in China (Fa, 1993), Indonesia (Parede, 1992), Vietnam (Vui *et al.,* 2002) and Ecuador (Hernandez-Divers *et al.,* 2006). IBD was also reported from several countries in Africa, including Zimbabwe (Kelly *et al., 19*94), Niger (Idi *et al.,* 1999), Kenya (Ndanyi, 2005), Egypt (Azzam *et al.*, 2004; Hassan, 2004), Mauritania (Bell *et al.,* 1990) and Botswana (Mushi *et al.,* 2006). The disease was first diagnosed in Ethiopia in 2002 in commercial poultry (Zeleke *et al.,* 2005a), and thereafter in a government-owned poultry multiplication centre (Woldemariam and Wossene, 2007) and a commercial broiler farm (Chanie *et al.,* 2009). In Ethiopia, IBD in backyard chickens has been serologically documented from the northwest and central part of the country (Mazengia *et al.,* 2009, Degefu *et al.,* 2010).

Mycoplasma gallisepticum (MG), together with *Escherichia coli*, is the cause of chronic respiratory disease (CRD) in chickens and is the most economically important of the avian *Mycoplasma* spp. (Bradbury, 2001). Mycoplasmas are also well known for their interactions with other infectious agents, such as ND virus, and environmental factors in producing clinical disease (Kleven, 1998). The disease has been reported, by serology or isolation of the agent, in backyard poultry in a few African countries including Niger (Idi *et al.,* 1990), Zimbabwe (Kelly *et al.,* 1994), Botswana (Mushi *et al.,* 1999), Benin (Chrysostome *et al.,* 1995) and Kenya (Ndanyi, 2005), as well as from Ecuador (Hernandez-Divers *et al*., 2006) and Argentina (Xavier *et al.*, 2011), and in fancy breed poultry flocks in Switzerland (Wunderwald and Hoop, 2002) where the management system is equivalent of the one in backyard poultry flocks. In Ethiopia, the disease was reported recently and MG was isolated from commercial broiler farms (Chanie *et al.*, 2009). No reports could be found of its presence in backyard chickens in Ethiopia.

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Fowl cholera, caused by *Pasteurella multocida* (PM), is another disease of significant economic importance with a worldwide distribution (Christensen and Bisgaard, 2000). The characteristic signs of the disease are respiratory rales, coughing and nasal discharge. Fowl cholera is considered a leading killer of domestic and wild birds in Asia (Rimler and Glisson, 1997). However, literature on the epidemiology and significance of infections caused by PM in poultry in developing countries is scanty, with reports in backyard chickens in Thailand (Thitisak *et al*., 1989), Zimbabwe (Kelly *et al.,* 1994), Tanzania (Muhairwa *et al.,* 2001) and Kenya (Mbuthia *et al.,* 2008). There are no published reports of this disease in either commercial or scavenging backyard chickens in Ethiopia.

The paucity of information on the presence and prevalence of the above diseases in backyard chickens may reflect a lack of resources for disease surveillance and control in backyard production systems. In addition, the diagnostic coverage of poultry diseases in Ethiopia is limited to the extent that, even from commercial farms, only a few cases are brought to either the National Animal Health Diagnostic and Investigation Center (NAHDIC) or the National Veterinary Institute (NVI). Most poultry outbreaks, particularly in more remote parts of the country, remain undiagnosed and dead chickens are simply discarded. Therefore, information on the prevalence and significance of infectious poultry diseases can only readily be obtained through indirect serological studies on apparently healthy chickens. It is difficult to design and implement chicken health development programs without an understanding of the diseases present in the backyard poultry production system. Hence this study was implemented to determine the seroprevalence of ND and other major poultry diseases potentially affecting backyard poultry health in Ethiopia, to determine the level of concurrent seropositivity to multiple pathogens, and to determine any seasonal or geographic patterns of seroprevalence.

2.3 Materials and methods

2.3.1 Study area, study design and sample size

Administratively, Ethiopia is divided into regions which are again sub-divided into zones and then woredas (districts). The study was conducted in the Eastern Shewa zone of the Oromia Region, in the woredas of Adami-Tulu-Jido-Konbolcha (ATJK) and Ada'a. In order to have animals coming from a diversity of sources, four rural markets (two from each woreda) were purposely targeted. Accordingly, Dire and Tuludimitu markets in Ada'a woreda and Adami

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Tulu and Bulbula markets in ATJK Woreda were selected.

A cross-sectional serological survey was carried out twice during 2010: in January, representing the dry season, and in September, representing the wet season.

Since various prevalences had been reported for ND virus antibodies in previous studies, ranging from 11% (Regasa *et al.,* 2007) to 38% (Tadesse *et al.*, 2005) an expected seroprevalence of 20% was assumed for ND. Since little or no information was available for the other diseases, the same expected seroprevalence as for ND was assumed. The sample size was therefore calculated in order to estimate a prevalence of 20% with 95% confidence and 10% absolute error; this gave a required sample size of 62 birds for each market, during each season. On each sampling occasion a market was visited twice, on two consecutive market days. Apparently healthy chickens of local breed, greater than 2 months of age, were purchased (only one chicken from an individual seller), individually identified using a leg band and transported to NAHDIC. In addition, individual farmer-sellers were interviewed regarding chicken disease occurrence during the previous six months (with clinical signs including mass mortality, respiratory distress, diarrhoea, ocular and nasal discharges, or nervous signs) and vaccination history in their village of origin.

2.3.2 Sampling procedure and sample analysis

Immediately after arrival at the NAHDIC laboratory, blood samples were collected from the brachial vein in 3 mL disposable syringes and left horizontally for 3 hrs and then vertically for the serum to ooze out. Serum was collected in 2 mL cryovial tubes and kept at -20°C until testing.

Serum samples were analyzed using commercial ELISA kits for the presence of antibodies to ND (Svanovir NDV-Ab Elisa, Svanova Veterinary Diagnostics, Sweden), MG (*Mycoplasma gallisepticum* Ab test kit, Svanova Veterinary Diagnostics, Sweden), IBD (LSI IBD ELISA, Laboratoire Service International, France), and PM (Synbiotics ProFLOK®*Pasteurella multocida*, Synbiotics Corporation, U.S.A.), according to the manufacturers' instructions.

The ND virus antibody and MG ELISAs work on the principle of blocking ELISA and are developed to detect specific antibodies against APMV-1 and MG, respectively, in serum. The sample and control optical density (OD) values were read using an ELISA reader

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(Immunoscan, BDSL) at 450 nm. From OD values percent inhibition (PI) was calculated for control and test samples using the following formula:

PI = [(OD negative control– OD sample)/OD positive control] x 100/OD negative control

The LSIVET IBD and ProFLOK® PM antibody ELISA are based on the principle of indirect ELISA. The samples and controls OD values were read using an ELISA reader (Immunoscan, BDSL) at 405 nm. For each sample the sample-to-positive (S/P) ratios were calculated from OD values by the formula:

S/P ratio= [Sample OD– negative control mean OD]/ [Positive control mean OD– negative control mean OD].

2.3.3 Data analysis

Data from the laboratory analyses were stored in a spread sheet, and PI or S/P values were computed as above. The seroprevalence of each disease, for each market, woreda and season, with binomial exact 95% confidence intervals, were calculated. Seroprevalence was compared between woredas and seasons using Fisher's exact test. The median number of diseases for which birds were seropositive was compared between seasons using the Wilcoxon rank-sum test. A significance level of $\alpha = 0.05$ was used. Analyses were done using STATA 11 (Stata Corp, College Station, Texas, U.S.A.)

2.4 Results

Serum samples from a total of 250 and 229 apparently healthy chickens were collected during the dry and wet seasons, respectively. However, only 234 and 216 sera, respectively, were analyzed because of the limited quantity and quality of serum obtained from some of the birds.

From the interviews we learned that none of the farmer-sellers had ever vaccinated their chickens. However, 23.4% and 30% of sellers, during the dry and wet seasons respectively, reported to have had disease amongst their poultry flocks during the previous six months, with a combination of signs including depression, inappetance, diarrhea, respiratory distress and paralysis in some cases.

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Table 2.1 shows the prevalence of ND, PM, MG and IBD antibodies in the different markets and woredas during the dry season. The seroprevalence of ND ranged from 4.3% (Bulbula) to 7.8% (AdamiTulu), but no significant difference was seen between woredas. A high ELISA PI values (as high as 94) were recorded for some of the positive individual animal sera. For PM the seroprevalence varied from 56.8% (Tuludimitu) to 78.5% (Bulbula) and was significantly higher in ATJK (72.4%) than in Ada'a (58%) woreda (P =0.025). The seroprevalence of MG had a wider variation, from 32.4% (Tuludimitu) to 67.2% (AdamiTulu) and was also significantly higher in ATJK (63.4%) than in Ada'a (50%) woreda (P =0.045). IBD seroprevalence was high in all the markets, and did not differ significantly between woredas.

Table 2.1 Seroprevalence of Newcastle disease (ND), *Pasteurella multocida* (PM), *Mycoplasma gallisepticum* (MG) and infectious bursal disease (IBD) in backyard chickens at markets in Eastern Shewa zone, Ethiopia, during January 2010 (dry season).

Woreda/market	n	Percent seropositive (exact 95% C.I.)			
		ND	PM	MG	IBD
ATJKWoreda	134	5.9 (2.6;10.0)^{A}	72.4 (64;79.5)^{B}	63.4 (54.6;72.0)^{B}	94.8 (89.5;98)^{A}
AdamiTulu	64	7.8 (2.6;17.4)^{a}	65.6 (52.6;77.0)^{a}	67.0 (54.3;78.4)^{a}	100 (94.3;100)^{b}
Bulbula	70	4.3 (0.9;12.0)^{a}	78.5 (67.1;87.5)^{a}	60.0 (47.6;71.5)^{a}	90.0 (80.4;95.9)^{a}
Ada'aWoreda	100	6.0 (2.2;12.6)^{A}	58.0 (47.7;67.8)^{A}	50.0 (39.8;60.2)^{A}	88.0 (79.9;93.6)^{A}
Dire	63	6.3 (1.8;15.5)^{a}	58.0 (45.6;71.0)^{a}	60.3 (47.2;72.4)^{b}	87.3 (76.5;94.4)^{a}
Tuludimitu	37	5.4 (0.6;18.2)^{a}	56.8 (39.5;73.0)^{a}	32.4 (18.0;49.8)^{a}	89.2 (74.6;97.0)^{a}
TOTAL	234	5.9 (3.3; 9.8)	66.2 (59.7;72.0)	57.7 (51.1;64.0)	91.9 (87.6;95.0)

^{A,B}Woredas with different superscripts differ significantly (P< 0.05)

^{a,b}Within Woreda, markets with different superscripts differ significantly (P< 0.05)

Table 2.2 shows the prevalence of ND, PM, MG and IBD antibodies in the different markets and woredas during the wet season. The seroprevalence of ND showed wider variation between markets than during the dry season, ranging from 3.2% (Bulbula) to 10.3% (Tuludimitu). No significant difference was seen between woredas but a relatively lower

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proportion of seropositive chickens were recorded in ATJK (4.2%) than in Ada'a (8.0%) woreda. For PM the prevalence varied from 60.3% (Dire) to 72.4% (Tuludimitu). Fairly similar seroprevalence (63%) was recorded between woredas. The seroprevalence of MG varied from 66.1% (Dire) to 87.5% (AdamiTulu) and was significantly higher (P=0.007) in ATJK (85.7%) than in Ada'a (70.1%) woreda. During the wet season IBD seroprevalence was closely similar between woredas: 96.6% in ATJK and 95.9 % in Ada'a, and it did not differ significantly between markets.

Table 2.2 Seroprevalence of Newcastle disease (ND), *Pasteurella multocida* (PM), *Mycoplasma gallisepticum* (MG) and infectious bursal disease (IBD) in backyard chickens at markets in Eastern Shewa zone, Ethiopia, during September 2010 (wet season).

Woreda/market	n	Percent seropositive (exact 95% C.I.)			
		ND	PM	MG	IBD
ATJK Woreda	119	4.2 (1.4;9.5)^{A}	63.0 (53.7;71.7)^{A}	85.7 (78;91.4) ^{B}	96.6 (91.6;99)^{A}
AdamiTulu	56	5.4 (1.1;15.8)^{a}	62.5 (48.5;75.0)^{a}	87.5 (76;94.8)^{a}	94.6 (85.1;98.8)^{a}
Bulbula	63	3.2 (0.4;11.0)^{a}	63.4 (50.4;75.3)^{a}	84.1 (72.7;92.1)^{a}	98.4 (91.5;100)^{a}
Ada'a Woreda	97	8.0 (3.6;15.6)^{A}	63.9 (53.5;73.4)^{A}	70.0 (59.9;78.9) ^{A}	96.0 (89.7;98.8)^{A}
Dire	68	7.4 (2.4;16.3)^{a}	60.3 (47.6;72.0)^{a}	66.1 (53.6;77.2)^{a}	94.1 (85.6;98.4)^{a}
Tuludimitu	29	10.3 (2.2;27.4)^{a}	72.4 (52.7;87.3)^{a}	79.3 (60.3;92)^{b}	100 (88.1;100)^{a}
Total	216	6.0 (3.2;10.0)	63.4 (56;67.0)	78.7 (72.6;84.0)	96.3 (93;98.4)

^{A,B}Woredas with different superscripts differ significantly ($P < 0.05$)

^{a,b}Within Woreda, markets with different superscripts differ significantly ($P < 0.05$)

During both wet and dry seasons a fairly similar proportion (6%) of chickens had antibodies against NDV. But during both seasons the proportion of chickens which were seropositive for PM, MG and IBD could be considered high, reflecting the widespread prevalence of those diseases. Both overall and within woredas the seroprevalence of MG was significantly higher during the wet season than during the dry season ($P < 0.001$).

The distribution of the number of diseases under investigation to which an individual bird was concurrently seropositive is shown in Figure 2.1. It was found that 81.2% and 91.2% of

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the chickens tested had antibodies to at least two of the diseases under investigation during the dry and wet seasons, respectively. The median number of diseases to which the birds were seropositive during the dry and wet seasons were 2 and 3, respectively, which differed significantly (P =0.002). Overall, less than 1% of the chickens were seronegative for all four diseases, while 2.67% had antibodies to all four diseases investigated. Most of the concurrent seropositivity (>75%) was due to IBD with either MG or PM, or both. No bird was seropositive for ND alone.

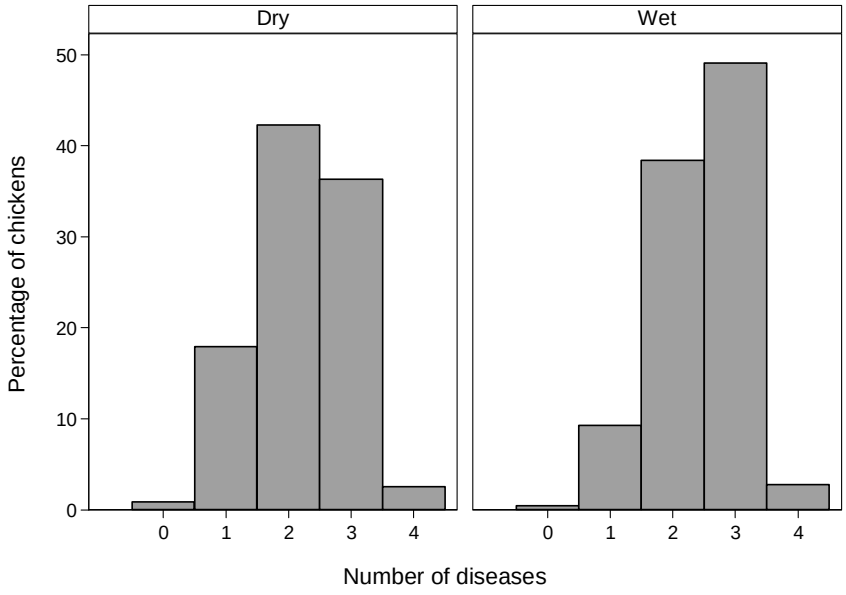

Figure 2.1 Seasonal distribution of the number of diseases to which an individual bird was seropositive, in backyard chickens at markets in Eastern Shewa Zone, Ethiopia, tested for antibodies to Newcastle disease virus, *Pasteurella multocida*, *Mycoplasma gallisepticum* and infectious bursal disease virus.

2.5 Discussion

In unvaccinated flocks, positive serological results are clear evidence that the birds have been exposed to the infectious agent under investigation, although without identifying the infecting strains. In the present study we confirmed from the sellers during purchase that none of them had vaccinated their chickens for any poultry diseases. Hence, the presence of antibodies to ND, PM, MG and IBD was considered evidence of exposure to natural infection to the respective disease-causing agent.

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The study revealed that the prevalence of ND antibodies in backyard chickens was generally low, around 6%. This is considerably lower than previous reports by Zeleke *et al*. (2005b) and Tadesse *et al*. (2005), who reported prevalences of 19.8% in the southern and Rift Valley districts and 32.2%in central Ethiopia, respectively, but closer in magnitude to that reported by Regasa *et al*. (2007) in southern Ethiopia (11%). The results are also consistent with seroprevalences in backyard poultry of 4.8% in Mauritania (Bell *et al*., 1990), 2.2% in Mexico (Gutirrez-Ruiz *et al*., 2000), 4.8% in California (McBride *et al.,* 1991) and 5% in South Africa (Thekisoe *et al*., 2003). When chickens are affected by a velogenic ND virus that results in very high or 100% mortality, one is likely to find few or no survivors with antibodies. Up to 30% of market sellers claimed to have observed poultry disease signs (sudden death, diarrhea and nervous signs) resembling Newcastle disease clinical signs during previous months. Moreover, the fact that high ELISA PI values (as high as 94) were recorded for some of the positive individual animal sera, in the absence of vaccination, suggests that velogenic virus outbreaks might have killed most chickens in the villages and left few survivors with high antibody titre (Chrysostome *et al*., 1995; Alexander *et al.,* 2004). According to Awan *et al*. (1994), low ND HI antibody prevalence is suggestive of an interepidemic phase and this could partly explain the high proportion of seronegative chickens in the present study. The seroprevalence was far lower than that reported from Ecuador (97%) (Hernandez-Divers *et al.,* 2006), Tanzania (46.1%) (Yongol *et al.,* 2001), Zambia (36.9%) (Alders *et al.,* 1994), Zimbabwe (27%) (Kelly *et al.,* 1994) and Bangladesh (88%) (Biswas *et al.,* 2009). The discrepancy could be explained by differences in study settings, or by exposure to mild virus strains that induced immunity but did not kill many chickens. The presence of lentogenic, or possibly mesogenic ND in backyard chickens in an area may result in a constant cycle of infection which, from time to time, boosts the immunity of exposed chickens resulting in a higher proportion of chickens with antibodies (Sagild and Haresnape, 1987; Martin, 1992). Another reason for variation in the results between studies could be subjectivity and variation in HI cut-off values used for the interpretation of the result. For instance some authors considered a HI titre $\geq 1\log_2$ as positive (Alders *et al*., 1994; Chrysostome *et al*., 1995; Biswas *et al.,* 2009), while others used cut-off titres of $3\log_2$ (Zeleke *et al*., 2005b; Tadesse *et al*., 2005) or of $4\log_2$ (Gutierrez-Ruiz *et al*., 2000). Given the periodic outbreaks and probable high mortality amongst birds affected with ND, our serological findings are likely to be a reasonable indication of the level of ND virus antibody in individual backyard chickens. There was no observed seasonal or geographic variation in

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individual seroprevalence as such, in the present study, suggesting that the disease is widespread and occurs throughout the year in the study area.

The study revealed a high seroprevalence of fowl cholera (65%) in backyard chickens in the study area, and this constitutes the first report of fowl cholera seroprevalence in Ethiopia. The present finding is in close agreement with that of Kelly *et al.* (1994), who documented a prevalence of 52% in backyard chickens in Zimbabwe. The high prevalence may be due to infection of backyard chickens by less virulent strains, with or without any clinical signs or any significant mortality. Such a phenomenon has also been described after challenge with a low virulence strain causing signs of chronic fowl cholera in Kenya (Mbuthai *et al.,* 2008). Biswas *et al.* (2005) reported proportional mortality from PM of only 6.7% from a longitudinal study in free-range scavenging chickens' in Bangladesh. Mortality is not a typical outcome of fowl cholera in backyard chickens but it may decrease feed efficacy (Mbuthia *et al.,* 2008). Hence there is a high probability that infected birds will seroconvert and remain convalescent carriers, explaining the observed high prevalence (Muhairwa *et al.,* 2000). Surviving birds from diseased flocks may therefore be a risk to naïve birds. Investigations indicate that carriers of PM may exist within poultry flocks with or without a history of previous outbreaks of fowl cholera (Christensen and Bisgaard, 2000; Mbuthia *et al.*, 2008). Mbuthia *et al*., (2008) were able to isolate PM from 6.2% of healthy-looking chickens from free-range family poultry farms and at market slaughter slabs in Kenya. Sharing drinking water and feed, which is the common practice in backyard poultry systems in Ethiopia, can facilitate transmission of the bacteria between birds. Chickens may then suffer slight clinical disease and develop immunity, resulting in a high seroprevalence.

The MG prevalence observed in this study averaged 67.7%. This high prevalence is in close agreement with reports from Benin (62%) (Chrysostome *et al*., 1995), Botswana (57.8%) (Mushi *et al.,* 1999), South Africa (63%) (Thekisoe *et al.,* 2003), Ecuador (73%) (Hernandez-Divers *et al.,* 2006) and Bangladesh (58.9%) (Sarkar *et al.,* 2005). Relatively lower seroprevalence was reported in village chickens in Malaysia (18.6 - 25.7%) (Faisal *et al.*, 2011; Shah-Majid, 1996) and Zimbabwe (<33%) (Kelly *et al*., 1994). Seasonal variation of MG seroprevalence was observed in our study, with higher seroprevalence observed during the wet season (78.7%) than during the dry season (57.7%). This could be due to cold and wet conditions that stress the birds and make them more susceptible to respiratory infections. Sarkar *et al.* (2005) also found that MG had a seasonal pattern in Bangladesh, where the

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seroprevalence was higher during winter than summer. Seroprevalence of MG was also higher in ATJK (74%) than in Ada'a (60%) woreda. This could be associated with the more dusty nature of the areas in ATJK than in Ada'a, as well as to differences in hygienic conditions. Lack of cleaning and hygienic practices in chicken houses may result in ammonia build-up in the wet season that predisposes to respiratory system infection such as mycoplasmosis (Johnson, 1983). However, this was not assessed in the present study. The occurrence of a MG outbreak in commercial broiler chickens in Ethiopia was associated with overcrowding, poor housing, poor sanitation and changes in environmental factors (Chanie *et al*., 2009). Mycoplasmas are also well known for their interactions with other infectious agents and environmental factors in producing clinical disease (Kleven, 1998). Hence, it appears that mycoplasmas, along with other bacterial or viral pathogens, may be responsible for a considerable proportion of the respiratory signs that were reported by rural communities in Ethiopia.

The survey also indicated that IBD is widespread among village chickens in the study area, with a seroprevalence of 94%. This agrees closely with reports by Degefu *et al*.(2010) from Ethiopia, Hernandez-Divers *et al.* (2006) from Ecuador, Idi *et al*. (1999) from Niger and Karunakaran *et al.* (1993) from India, who reported seroprevalences of 76.6%, 100%, 74% and 73.7%, respectively. But relatively lower IBD seroprevalences were recorded in Mauritania (15.8%) (Bell *et al.,* 1990), Zimbabwe (55%) (Kelly *et al.,* 1994), Kenya (49.3%) (Ndanyi, 2005) and Botswana (66.2%) (Mushi *et al*., 2006). The higher seroprevalence of the disease in the study area, in the apparent absence of mortality, could be due to an IBD virus of lower pathogenicity, unlike the case reported from the Amhara region of Ethiopia (Mazengia *et al.,* 2009) or the outbreak in a commercial broiler farm with evident mortalities (Zeleke *et al.,* 2005a). It is also possible that the birds were infected with IBD virus as adults, at which stage they simply seroconvert without any apparent clinical disease. With such a high seroprevalence and low mortality of infected birds, there is the possibility of genetic resistance amongst indigenous breeds of chickens in Ethiopia, as reported from Egypt (Hassan *et al.,* 2004). This is difficult to demonstrate using serological studies, but further studies could be undertaken to investigate this finding.

In general, there was very little variation between seasons in the seroprevalence of PM, IBD and ND in this study, suggesting the endemicity of those diseases throughout the year in backyard chickens. But variation in the seroprevalence of PM, MG, and IBD was observed

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between areas during the dry season of the year, suggesting that there could be variation in disease incidence that could be explained partly by variation in local conditions and variation in age structure of flocks in different areas. This could be further substantiated with longitudinal studies that should also take into account the various potential confounders.

In this study a high percentage of concurrent seropositivity to multiple infectious agents was observed. Although it could not be determine whether there had been concurrent infection, most of these involved the immunosuppressive IBD. Although the exact role of each disease is not very clear, the fact that the vast majority of chickens tested had antibodies to two or more of the diseases under investigation, suggests that their health was impacted by multiple infectious pathogens. A similar observation was made in a commercial production system in Ethiopia (Chanie *et al*., 2009). Biswas *et al.* (2009) in a serological investigation had reported the existence of various viral diseases in smallholdings in Bangladesh. Bell *et al*. (1990), in an investigation of the disease status of village chickens in Mauritania, confirmed serologically that village chickens were exposed to NDV, IBD and *Salmonella pullorum* to varying degrees. Similarly a study from Zimbabwe demonstrated that backyard chicken flocks had been exposed to several viral and bacterial diseases (Kelly *et al.*, 1994). Biswas *et al.* (2005) reported the existence of a combination of different viral and bacterial diseases in small holdings in Bangladesh and concluded that death was the synergic cooperation between or among different pathogens. Hence, it is possible that the mortalities amongst backyard chickens in Ethiopia could be explained not only by periodic occurrences of velogenic NDV, but also by the synergic effect of other disease-causing agents. Synergism has been demonstrated between MG, ND and infectious bronchitis (Bradbury, 1984). Carpenter *et al.* (1991), in a study of turkey flocks, indicated that flocks that had antibodies to ND virus and/or *Mycoplasma meleagridis* had an increased risk of having an outbreak of fowl cholera. It is generally believed that concurrent infection renders backyard chickens more susceptible to ND infection (Martin, 1992). The fact that mortality was high even in ND vaccinated flocks indicates that mortalities are the synergic effect of other concurrent infections as well (Kyvsgaard *et al.,* 1999). Kleven (1998) concluded that control of the clinical manifestations of mycoplasma infections is simplified when concurrent infections are minimized and optimum environmental conditions are provided. However, a serological study such like this one cannot provide an accurate indication of concurrent infection of ND with other diseases, firstly since most chickens might have died due to virulent strains of ND, and secondly because serology cannot indicate precisely when the infectious agent was present.

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Nevertheless, the high prevalence of concurrent seropositivity, and the fact that about 2.5% of the chickens had antibodies to all the four diseases suggests that backyard chickens are under constant pressure due to several infectious diseases.

In conclusion, the present study revealed that several infectious poultry diseases are widespread in backyard chickens in the study area. These diseases are considered to have a significant economic implication individually and/or concurrently. It is also likely that other infections occur in addition to the ones investigated (Tadesse *et al.,* 2004). As serology is not able to demonstrate which strains are circulating, further work is recommended to better understand the circulating strains or pathotypes and the epidemiology of these diseases. Secondly, improvement of village chicken production is at least partly dependent on successful control of some or all of those diseases. Further study is necessary to understand the interactions of these infectious poultry diseases and to estimate their impact on the backyard poultry production system.

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CHAPTER THREE

SEROLOGICAL AND MOLECULAR INVESTIGATION OF NEWCASTLE DISEASE IN HOUSEHOLD CHICKEN FLOCKS AND ASSOCIATED MARKETS IN EASTERN SHEWA ZONE, ETHIOPIA

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3.1 Abstract

A cross-sectional survey for Newcastle disease (ND) was conducted in non-vaccinated household flocks of village chickens to assess the serological and virological ND status in households and associated live bird markets. In total 1899 sera and 460 pools of cloacal and tracheal swabs were sampled and tested using a commercial enzyme-linked immunosorbent assay (ELISA) and real time reverse transcriptase polymerase chain reaction (rRT-PCR), respectively. Additionally, paired cloacal and tracheal swabs from 1269 individual chickens were collected from markets and tested using RT-PCR. The prevalence of households with at least one seropositive chicken was higher during the dry season (27.4%) than during the wet season (17.4%) (P = 0.003). Viral genome was detected in 14.2% of households during the wet season using a fusion (F) gene assay and in 24.2 % of households during the dry season using a polymerase (L) gene assay that targets both class I and class II viruses. At the markets sampled overall bird level prevalence was 4.9% for period 1 (F gene assay), and 38.2% and 27.6% for periods 2 and 3, respectively (L gene assay). Partial sequencing of the F gene (239 bp) cleavage site indicated that the majority of the circulating strains exhibited motifs specific to virulent strains. Seroepidemiology coupled with molecular analysis can be a useful tool to assess the status of NDV infection. The village chicken population in Ethiopia is endemically infected with virulent NDV that pose a significant threat to emerging small and medium scale commercial poultry production.

Key words: Newcastle disease; village chickens; ELISA; rRT-PCR; Ethiopia

3.2 Introduction

Newcastle disease (ND) is caused by avian paramyxovirus serotype 1 (APMV-1) belonging to the family *Paramyxoviridae*, genus *Avulavirus* (Mayo, 2002). Newcastle disease virus (NDV) can be categorized into highly pathogenic (velogenic), intermediate (mesogenic), and less-pathogenic (lentogenic) strains based on pathogenicity in chickens, and are divided in two clades (class I and class II) (Czegledi *et al*., 2006). Class I contains almost exclusively low virulence strains recovered from wild waterfowl worldwide. Class II includes strains of low and high virulence isolated from poultry and wild birds. The disease is endemic in the village poultry population in Africa (Bell *et al*., 1990; Echeonwu *et al*., 1993; Awan *et al.,* 1994; Chrysostome *et al*., 1995; Orjaka et al., 1999; Abolnik *et al*., 2004; Otim *et al*., 2004;

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Zeleke *et al*., 2005b; Servan de Almeida *et al.,* 2009; Snoeck *et al*., 2009; Cattoli *et al*., 2010, Rasamoelina Andriamanivo *et al.,* 2012) and is regarded as the most important constraint to the development, survival and productivity of village chicken flocks (Alexander *et al*., 2004; Alders, 2009).

Newcastle disease was first reported in 1971 from a small poultry farm in Asmara, then part of Ethiopia (Bawke *et al*., 1991), and since then has been considered the most devastating disease of chickens. From the limited reports to the World Organisation for Animal Health (OIE) it also appears to be the most important poultry disease in Ethiopia, recurring every year (http://web.oie.int/wahis/public.php). However, published information, either serological or virological, on ND epidemiology in Ethiopia is limited. A few cross-sectional studies in backyard chickens have showed that the disease is present with reported individual seroprevalences ranging from 5.6% to 38% (Zeleke *et al*., 2005b; Tadesse *et al*., 2005; Getachew, 2009; Chaka *et al*., 2012a). Epidemiological and molecular investigation to understand the NDV status of backyard chickens has never been done in Ethiopia. Molecular analysis, along with further characterisation of NDV field isolates, would be useful in order to know the type of strains circulating in a particular geographical area. Reverse transcriptase polymerase chain reaction (RT-PCR) is a useful tool to achieve this without propagation in embryonated eggs (Singh *et al*., 2005), allowing subsequent sequencing of the amplified DNA and pathotyping even if the virus is found in minute quantities or has lost infectivity (Seal *et al.*, 1995; Gohm *et al.*, 2000; Singh *et al.*, 2005). The pathotype can be characterized by the amino acid sequence of the cleavage site of the fusion (F) protein (Aldous *et al.*, 2003; de Leeuw *et al*., 2005).

The objectives of this study were to determine the seroprevalence at household level (using ELISA) and the prevalence of NDV-infected household flocks (using RT-PCR), during both wet and dry seasons. A further objective was to sample selected live bird markets in order to investigate the prevalence of NDV-infected birds using RT-PCR.

3.3 Materials and methods

3.3.1 Study area

Administratively, Ethiopia is divided into regions which are again sub-divided into zones and then into woredas (districts). A woreda is an administrative unit composed of kebeles, each

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containing several garees (villages). The study was undertaken in the Oromia administrative region, among households in two randomly selected woredas of East Shewa zone, namely Ada'a and Adami Tulu Jido Kombolcha (ATJK) (Figure 3.1). The East Shewa zone was specifically selected for its relatively high poultry and human population density in the region and the presence of wetlands where other complementary studies were also undertaken. Six markets in the selected woredas were also included for molecular investigation: Hidi, Godino, and Bakajo in Ada'a woreda, and Adamitulu, Bulbula, and Abosa in ATJK woreda.

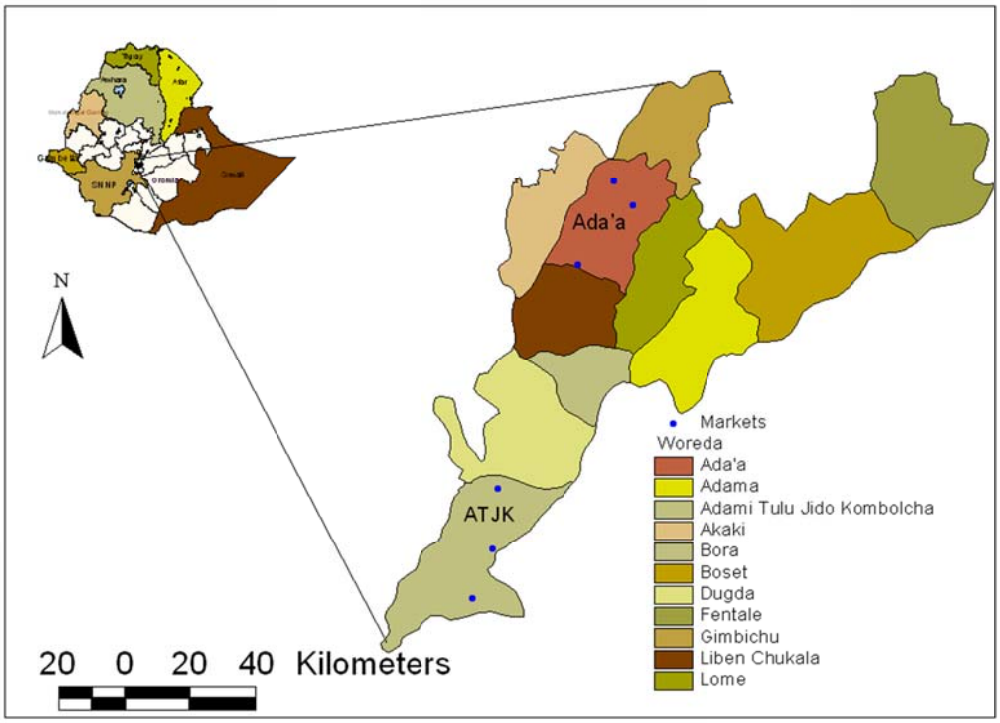

Figure 3.1 Map of East Shewa zone, Ethiopia indicating the two selected woredas and markets.

3.3.2 Study design and sample size

A cross-sectional survey was conducted amongst household flocks, with repeated sampling of the same households in September 2009 and May 2010, corresponding to the wet and dry seasons, respectively. The unit of interest was a household flock. The number of households to be sampled was determined at an expected prevalence of 35%, allowable error of 10% and confidence level of 95%, with a design effect of 3 to account for the multistage survey design (Bennett *et al*., 1991; Gutierrez-Ruiz *et al*., 2000), giving a required sample size of 264

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households. First, a list of kebeles in each woreda was compiled and two kebeles were randomly selected from each. In each selected kebele a list of garees was compiled and 12-14 were randomly sampleddepending on resource availability. Within each garee, five households were randomly selected, provided at least four chickens were present and there was no history of vaccination. Random sampling processes at different stages was attained using the "sample" command in STATA 8 (Stata Corp, College Station, TX, U.S.A) that helps to draw random sample from the lists provided. From each selected household flock four chickens over 8 weeks old were 'randomly' captured by the owners after putting in a house, since formal randomization was difficult to attain, and sampled . During the second sampling the same households were sampled; where fewer than four chickens were present, all were sampled. Farmers were also asked whether any new additions of chickens had been made to their flocks from outside sources (either from markets or as a gift) since the first sampling, and whether any signs suggestive of ND infection (respiratory distress, diarrhea, ocular and nasal discharge, nervous signs or sudden death) had been noted in their flocks.

The six markets were strategically selected, being markets that served the study woredas, and where birds sold were only from producer-sellers. The number of birds to be sampled in each market was calculated assuming a market size of 50-100 chickens, a minimum expected prevalence of 5% and a 95% confidence level. Sellers were selected by convenience sampling and only one bird was swabbed from any individual seller on each occasion. Market sampling was done between June 2009 and August 2010, during three strategically selected sampling periods: periods 1 and 3 representing wet seasons (June, July and August) and period 2 representing the dry season (March and April). Visit was made once to each market every month during each period.

3.3.3 Sampling procedures

In household flocks, 1.5 mL of blood was drawn from the brachial vein in a 3 mL disposable syringe, using a new syringe and needle for each chicken. Blood was allowed to clot and was kept at room temperature for serum collection. A total of 1018 and 881 individual chicken sera from 260 and 249 households were collected during the wet and dry seasons, respectively. For molecular investigation, tracheal swabs and cloacal swabs collected from all selected chickens in each household were pooled separately in viral transport media containing streptomycin sulphate, penicillin and mycostatin in phosphate buffer solution,

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resulting in 460 separate pools of each. The median number of samples per pool was 4 (3-4) and 4 (1-4) for wet and dry seasons, respectively. At the markets, tracheal and cloacal swabs were collected separately from individual chickens, resulting in 1269 pairs of swabs in total.

3.3.4 Serological testing

Enzyme-linked immunosorbent assays (ELISA) on chicken sera were performed at the National Animal Health Diagnostic and Investigation Center (NAHDIC), Sebata, Ethiopia, using a commercial blocking ELISA kit (Svanovir NDV-Ab, SVANOVA Biotech, Uppsala, Sweden) to detect specific antibodies against APMV-1 (Czifra *et al*., 1996; Gohm *et al*., 1999) according to the manufacturer's instructions. The sample and control optical density (OD) values were read using an ELISA reader (Immunoscan, BDSL) at 450 nm. The percentage inhibition (PI) was calculated from OD values according to the following formula:

$$PI = [(OD_{Negative\ control} - OD_{Sample}) / OD_{Positive\ control}] \times 100/OD_{Negative\ Control}$$

A household was classified as positive if one or more chickens in the flock tested positive, with PI > 40 based on manufacturer's recommendation.

3.3.5 RNA extraction and RT-PCR assay

Real time (r) RT-PCR assays were performed at the *Centre de Coopération Internationale en Recherche Agronomique pour le Développement* (CIRAD), Montpellier, France and at the Agricultural Research Council - Onderstepoort Veterinary Institute (ARC-OVI), Pretoria, South Africa. The swab samples from the first household sampling and the first market survey were tested at CIRAD to detect the NDV genome-specific fusion (F) gene cleavage site sequence. Viral RNA was extracted on a high throughput automated workstation Biomek FX^P (Beckman) using the Nucleospin RNA virus kit (Macherey Nagel). NDV was detected on the F gene by one step rRT-PCR method using a Stratagene Machine Mx3000 or 3005. Additionally, for the positive swab samples the coding region of the F gene (239 bp), including the cleavage site, was amplified and directly sequenced to determine the pathotype and to perform a phylogenetic study. Swab samples from the second household sampling and the second and third market surveys were tested at ARC-OVI. RNA was extracted using a Total Nucleic Acid extraction kit (Roche) on a MagnaPure robotic system (Roche), or using TriZol reagent (Invitrogen), according to the recommended procedure. The rRT-PCR method

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described by Fuller *et al.* (2010) was followed, using an LC480 thermocycler (Roche). Since this assay targets the polymerase (L) gene and thus detects a broader spectrum of APMV-1 (both class I and II viruses), 36 samples that tested NDV positive based on the L gene target were re-tested at ARC-OVI on an LC480 thermocycler (Roche) using the assay described by Wise *et al.* (2004) that targets a conserved region of the matrix (M) gene and detects only class II viruses. A household or bird (for market survey) was classified as positive if virus RNA was detected in either cloacal or tracheal swabs.

3.3.6 Data analysis

Data were managed in Microsoft Excel and analysed using STATA 11.1 (Stata Corp, College Station, TX, U.S.A). In all analyses two-tailed tests and a significance level of 5% were used. Estimates of the household-level seroprevalence of ND and viral genome prevalence, with 95% confidence intervals (CI), were calculated by season, woreda and kebele, adjusting for sampling weights in the multistage survey design. Sampling weight was calculated as the inverse of the probability that an individual household would have been selected in the survey. Prevalence was compared between woredas and kebeles using the Pearson chi-square statistic which was corrected for the survey design with the second-order correction of Rao and Scott (1984). For household samples, a hierarchical mixed-effects logistic regression model, with kebele and village as random effects, was used to compare the odds of seropositivity between seasons for household data. The association between seropositivity and rRT-PCR at the household level was assessed using odds ratios and the Fisher's exact test for each of the two sampling times, and agreement between the two tests was assessed using the kappa statistic. The prevalence of NDV genome detection in market samples was calculated with binomial exact 95% CI. The molecular results were not comparable between seasons because of the different assays employed.

3.4 Results

Overall, 1899 chicken sera (1018 and 881 during the wet and dry seasons, respectively) and 460 pairs of pooled swabs (243 and 217 during the wet and dry seasons, respectively) were tested in the household survey. Fewer samples were collected during the second sampling, mainly because 11 households no longer had any chickens present and in 24.1% (60/249) of the households fewer than four chickens were present. At the first sampling, flock size (excluding small chicks) ranged from 4 to 28 (median: 7; interquartile range (IQR): 5-10). At

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the second sampling, excluding households that had no chickens, flock size ranged from 1 to 36 (median: 6; IQR: 4-10). Table 3.1 shows the flock dynamics between the two sampling periods. Of the 70 households that contained seropositive birds during the second sampling, 75.1% (53/70) had reduced flock size. Of all the households, 55.8% (145/260) experienced reduced flock size, with loss due to disease reported by owners in 71.7% (104/145) of these, while in 34.6% (90/260) of all households the farmers reported to have had ND in their flocks in the period between the two samplings. Of the households where farmers reported chicken losses in their flocks, 60.5% (141/233) attributed it to disease, 15.5% (36/233) to predators, 4% (10/233) to climatic stress, 3.4% (8/233) to accidents and 16.3% (40/233) to a combination of these causes. Additionally, market off-take due to selling was reported in 90.4% (235/260) of the households.

Table 3.1 Household flock dynamics between September 2009 (wet season) and May 2010 (dry season) in village chicken flocks in Eastern Shewa zone, Ethiopia.

Flock change	Losses reported[a]	Serological status			Grand Total
		Pos (%)	Neg (%)	NS[c]	
Increased	Yes	5 (6.2)[b]	34 (42.0)		
	No	4 (4.9)	38 (46.9)		
	Tot	9	72		81
Decreased	Yes	47 (32.4)	49 (33.8)	8 (5.5)	
	No	6 (4.1)	32 (22.1)	3 (2.1)	
	Tot	53	81	11	145
Unchanged	Yes	8 (23.5)	15 (52.1)		
	No	0 (0)	11 (32.4)		
	Tot	8	26		34
	Grand total	70	179	11	260

[a] Losses of chickens reported by farmers due to diseases in their flocks.

[b] Cell percentage.

[c] Not sampled during May 2010 because of total losses of chickens.

ELISA PI values ≥60 were recorded in 80.4% (45/56) and 78.1% (75/96) of all seropositive chickens in 82.6% (38/46) and 78.6% (55/70) of seropositive households during the wet and dry season, respectively. In the market surveys a smaller number of swabs than expected were collected for some of the markets because very few chickens were present on the day of the visit. All farmers reported that their chickens had never been vaccinated.

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Table 3.2 shows the household-level seroprevalence of ND by woreda and kebele in the study area during the wet and dry seasons, adjusted for sampling weights. The prevalence of households with at least one seropositive bird was estimated to be 17.4% (95% CI: 7.8-34.2%) and 27.4% (95% CI: 14.3-46.1%) during the wet and dry seasons, respectively; this difference was statistically significant (OR=2.03, 95% CI: 1.3-3.2, *P*=0.003). During the wet season the household level seroprevalence was somewhat higher in Ada'a (20%) than in ATJK (15%) and varied from 11.5% to 22.3% among kebeles, although these differences were not statistically significant. Similarly, during the dry season the seroprevalence was relatively higher in Ada'a (34%) than in ATJK (21%) and varied from 17.6 % to 33.3% among kebeles. Of the 70 households that contained seropositive birds during the dry season, 70% (49/70) had previously tested negative during the wet season, suggesting new infections and seroconversion.

Table 3.2 Newcastle disease household flock seroprevalence during September 2009 (wet season) and May 2010 (dry season) in village chicken flocks in Eastern Shewa zone, Ethiopia.

Woreda/	Wet season			Dry season		
Kebele	n[a]	% pos (95% CI)	*P*-value	n[a]	% pos (95% CI)	*P*-value
ATJK woreda	130	15.2 (3.4-47.5)	0.48 (woredas)	127	21.5 (7.1-49.5)	0.16 (woredas)
Negalign	60	22.3 (17.7-27.7)	0.08 (kebeles)	59	28.8 (22.8-35.6)	0.09 (kebeles)
D/Abyata	70	11.5 (8.4-15.4)		68	17.6 (14-21.9)	
Ada'a woreda	130	20.0 (15.0-26.0)		122	34.5 (27.6-42)	
GGorba	70	20.0 (14.2-28.3)	0.81 (kebeles)	65	35.6 (28-43.9)	0.55 (kebeles)
Ude	60	17.4 (7.8-34)		57	33.3 (14.3-41.6)	
Overall	260	17.4 (7.8-34.2)		249	27.4 (14.3-46.1)	

[a] number of household flocks

Table 3.3 shows the rRT-PCR analysis results from the pooled swabs from household flocks. In the wet season, using the F gene assay, 14.2% (34/243) of households tested positive. In the dry season, using the L gene assay, 24.2% (51/217) tested positive. During the wet season the rRT-PCR prevalence was slightly higher in Ada'a woreda (18.7%) than in ATJK (9.3%) and a relatively higher prevalence was also observed in Ada'a during the dry season. However, these differences were not significant (Table 3.3).

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Table 3.3 Newcastle disease virus detection by molecular assays in household flocks during September 2009 (wet season) and May 2010 (dry season) in village chicken flocks in Eastern Shewa zone, Ethiopia.

Woreda/	Wet season[a]			Dry season[b]		
Kebele	n	% pos (95% CI)	*P*-value	n	% pos (95% CI)	*P*-value
ATJK woreda	118	9.9 (2.7-30.5)	0.39 (woredas)	127	20.41(7.7-44.1)	0.43 (woredas)
Negalign	60	6.3 (4.5-9.6)	0.12 (kebeles)	59	14.3 (1.1-18.7)	0.10 (kebeles)
D/Abyata	58	12.3 (8.6-17.3)		68	23.7 (18.9-29.2)	
Ada'a woreda	125	18.7 (1.7-75.1)		90	30.5 (5.0-78.5)	
GGorba	65	9.3 (5.9-14.4)	0.07 (kebeles)	65	22.8 (17.0-29.7)	0.08 (kebeles)
Ude	60	28.1 (21.2-36.2)		25	48.1 (35.2-61.3)	
Total	243	14.2 (3.2-45.2)		217	24.2 (12.0-42.8)	

[a] F gene assay for class I NDV.

[b] L gene assay for class I and II NDV (Fuller *et al*., 2010).

Table 3.4 shows the cross tabulation between household seropositivity and rRT-PCR result. There was a weak association during the wet season (OR = 2.14; 95% CI: 0.94, 4.89; P = 0.09) but not during the dry season (OR = 1.27; 95% CI: 0.63, 2.55; P = 0.58). The kappa statistic for agreement between the ELISA and the rRT-PCR was 0.12 and 0.05 during the wet and dry seasons, respectively, both of which can be considered as only slight agreement. In 77% (34/44) and 73% (41/56) of households which were seropositive by ELISA, during wet and dry seasons respectively, NDV genome was not detected by rRT-PCR. Seventy two per cent (175/243) and 57.6% (125/217) of households tested had neither detectable antibodies nor viral genome in their samples during wet and dry seasons, respectively.

Table 3.5 shows the molecular detection of NDV in cloacal and tracheal swabs sampled at markets during three different periods, between June 2009 and August 2010. The overall bird-level prevalence of class II NDV genome (F gene assay) during period 1 was 4.9%. Using the L gene-based assay that detects both classes of APMV-1 it was 38.2% and 27.6%, for periods 2 and 3, respectively. A weakly significant difference (P = 0.04) between woredas was only noted during period 2 (dry season), when the prevalence was higher in ATJK (42.2%) than in Ada'a (28.9%). There was no significant variation among markets within a woreda in any of the period except for markets in ATJK during period 1. Upon further analysis of 36 swabs from households and markets that were positive for the L gene assay (Fuller *et al*., 2010), only 14 (39%) were positive by the M gene assay that detects only class II NDV (Wise *et al*., 2004).

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Table 3.4 Associations between Newcastle disease serology and virus detection by molecular assays during September 2009 (wet season) and May 2010 (dry season) in village chicken flocks in Eastern Shewa zone, Ethiopia.

		Household rRT-PCR result					
		Wet Season[a]			Dry season[b]		
		Pos	Neg	Total	Pos	Neg	Total
Household serology	Pos	10 (4.1)[c]	34 (14.0)	46	15 (6.9)	41 (18.9)	56
	Neg	24 (9.9)	175 (72.0)	199	36 (16.6)	125 (57.6)	161
	Total	34	209	243	51	166	217
OR (95% CI)		2.14 (0.94-4.89)			1.27 (0.63-2.55)		
P-value		0.09			0.58		

[a] F gene assay for class I NDV.

[b] L gene assay for class I and II NDV (Fuller *et al*., 2010).

[c] Cell percentage.

Table 3.5 Bird-level prevalence of NDV genome at live bird markets in Eastern Shewa zone, Ethiopia between June 2009 and August 2010.

Woreda/ Market	Period 1 (June-August 2009)			Period 2 (March and April 2010)			Period 3 (July-August 2010)		
	n[1]	% pos (95% CI)	*P*-value	n[1]	% pos (95% CI)	*P*-value	n[1]	% pos (95% CI)	*P*-value
Ada'a woreda	282	5.3 (3.0;8.6)[A]	0.72	83	28.9 (19.5;39.9)[A]	0.04	87	33.3 (23.6;44.3)[A]	0.15
Hidi	107	1.9 (0.2;6.6)	0.09	15	20.0 (4.3;48.1)	0.5	22	36.4 (17.2;59.3)	0.92
Godino	25	4.0 (0.1;2.0)		16	37.5 (15.2;64.6)		7	28.6 (3.7-70.0)	
Bakajo	150	8.0 (4.2;13.6)		52	28.8 (17.1;43.1)		58	32.7 (21.0;46.3)	
ATJK woreda	440	4.5 (2.8;6.9)[A]		192	42.2 (35.1;49.5)[B]		185	24.9 (18.8;31.7)[A]	
A/Tulu	150	2.0 (0.4;5.7)	0.04	70	38.6 (27.1;51.0)	0.1	65	23.1 (13.5;35.2)	0.2
Bulbula	150	8.0 (4.2;13.6)		73	37.0 (26.0;49.1)		70	20.0 (11.4;31.3)	
Abosa	140	3.5 (1.2;8.1)		49	55.1 (40.0;69.3)		50	34.0 (21.2;48.8)	
Total	722	4.9 (3.4;6.7)		275	38.2 (32.4;44.2)		272	27.6 (31.3;45.4)	

[1] Number of chickens.

[A,B]Woredas with different superscripts differ significantly ($P < 0.05$).

On direct sequencing of partial F gene (239 bp) in rRT-PCR positive samples, the cleavage site showed amino acid motifs typical of velogenic strains ("G/RRQKR/FV", "G/RRRKR/FV", or "G/KRRKR/FV"). Preliminary results of phylogenetic analysis based on F gene sequences revealed that the virus strains fell in clusters within genotypes VI and VII.

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A few samples showed amino acid motifs characteristic of avirulent strains ("G/GRQGR/LI", "G/GKQGR/LI") from genotypes I and II of class II viruses. A complementary study involving detailed characterization by sequence analysis, of the virus isolates obtained at markets and households in the study area is presented in Chapter 5.

3.5 Discussion

The study has detected changes in ND seroprevalence in household poultry flocks over time thus indicating on-going exposure to NDV and incidence of the disease. The overall household level seroprevalence (proportion of household with at least one seropositive bird) was 17.4% and 27.4% during the wet and dry seasons, respectively, and was significantly higher during the dry season ($P = 0.003$). During the second sampling, corresponding to the dry season, fewer birds per household were found, indicating that households had lost their chickens, possibly due to incidence of the disease in their flocks, amongst other factors. This was corroborated in many cases by the farmer's report of clinical ND in their flocks, and 75.1% (53/70) of households seropositive during the second sampling had reduced flock sizes. Chicken off-take due to market selling, reported in 90.4% (235/260) of the households, also contributed to reduced flock size. In Nigeria, high seroprevalence has been demonstrated during November to March (dry season) indicating that more outbreaks occurred during that time of the year (Orajaka *et al*., 1999; Musa *et al*., 2009). Within seasons, although there was no significant difference in household-level seropositivity between woredas, but variation was observed among kebeles. This could indicate that, although the disease is ubiquitous, the epidemiology and ecology of NDV varies locally in terms of stages of epidemic cycle, where certain areas could be in an inter-epidemic period, having low virus activity, while others are in early infection or experiencing an active outbreak. Such phenomena, along with change in the village poultry population, with variable immune status, probably maintain NDV circulation throughout the year in backyard chickens.

The estimates for flock seroprevalence were comparable to the 15.9% (range 11.3 - 23.0%) reported from East Timor (Serrão *et al*., 2012), although a much higher flock seroprevalence (89%) was recorded in chickens of smallholdings in Bangladesh (Biswas *et al.,* 2009). The principal limitation in comparing the present finding with previous serological studies either in Ethiopia or elsewhere was that they mostly assessed individual bird antibody prevalence. In addition, most previous studies have employed haemagglutination inhibition (HI) tests

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with various cut-off values (Kelly *et al.*, 1994; Chrystome *et al.,* 1995; Idi *et al*., 1999; Tadesse *et al.*, 2005; Musa *et al.,* 2009). Variations in prevalence could also be attributed to ecological characteristics of a specific area, such as climate, settlement pattern, sanitary and socio-economic practices, which may facilitate disease spread, and also the season in which the study was done.

The ELISA PI values equal to or greater than 60 recorded in a high proportion of all seropositive chickens during both the wet season (82.6%) and the dry season (78.6%) in our study may suggest recent field exposure to virulent NDV strains (Chrysostome *et al*., 1995; Alexander *et al*., 2004). Velogenic strains of the virus are known to produce higher antibody titres than lentogenic and mesogenic strains (Luc *et al*., 1992; Alexander *et al*., 2004). Sequencing of the fusion protein cleavage site of rRT-PCR-positive household and market samples confirmed the circulation of virus strains bearing amino acid motifs "G/RRQKR/FV" or "G/RRRKR/FV", characteristic of virulent viruses (Lamb *et al*., 2007; OIE, 2008b). Since chickens had not been vaccinated, these were likely the strains causing most of the seroconversion in our study area, although the circulation of some avirulent strains from genotypes I and II, found in a few of our samples, may have contributed to this. It has previously been reported that velogenic NDV strains are common in village situations (Martin, 1992). Bell and Mouloudi (1988) observed that velogenic virus strains were common in village chickens in Morocco, and virulent strains were also observed in apparently healthy chickens in Mali and Madagascar based on rRT-PCR analysis (Servan de Almeida *et al*., 2009). Given that the chickens sampled in the present surveys were apparently healthy yet infected with virulent strains of NDV, this may suggest the possibility of carrier birds amongst the village chickens in Ethiopia. Such a phenomenon has been described by Awan *et al*. (1994) and has considerable implications in the epidemiology of the disease. Another interesting possibility is that circulating avirulent strains may be causing seroconversion and providing some degree of protection against virulent NDV, as has previously been reported (Turner *et al*., 1976; Spradbrow *et al*., 1980; Samuel and Spradbrow, 1989). It is also possible that some birds were sampled during the early stage of infection before any evident clinical signs of the disease.

Seropositivity was not highly correlated with virus RNA detection at household level, and agreement between the ELISA and rRT-PCR was poor, as revealed by very low kappa values. This could indicate that virus circulation is enhanced when the level of antibody

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wanes or the number of susceptible chickens in a flock increases. The presence or absence of detectable antibody levels within a flock depends also on the phase of infection at the time of sampling. Bell and Mouloudi (1988) in Morocco obtained more viruses from areas with lower antibody levels. In general, Ada'a woreda had the highest prevalence of NDV genome at household level during both seasons but with considerable variation, within woreda, among kebeles in the study area (Table 3.3). The reason for such variation, as for seroprevalence, may be due to local variation in the stages of the epidemic cycle, but variation in ecology between areas that can affect virus viability in the environment may also play a role (Orajaka *et al.*, 1999). From our field observations we also noted that households within villages in ATJK woreda were more scattered than those in Ada'a, which may have reduced the chance of contact transmission between household flocks during scavenging.

The molecular techniques employed for NDV detection varied between the two sampling periods; the L gene assay that detects viruses of all the genetic lineages in classes I and II showed a greater proportion of positives compared to the F gene-based assay that detects only class II viruses, and attributed as the potential causes for the such observed variations in proportions between samplings. This was further confirmed on the re-testing of a subset of the L gene-positive samples from the dry season at households and markets, where only about 39% of them were attributable to class II NDV. However, it is also possible that RNA degradation may have contributed to the lower proportion of positive samples upon repeated testing; therefore it is not possible to compare the estimated prevalences between the two samplings. Viral circulation may vary depending on factors that could increase the number of susceptible birds and predispose to outbreaks. These include a seasonal peak in hatching generally observed during the dry season in Ethiopia resulting from farmers' avoidance of wet season hatching with associated chick losses due to cold stress, as well as movements due to marketing practices. Increased movement of live birds during festivals, in anticipation of good prices, could increase the incidence of outbreaks (Sa'idu *et al.*, 2006; Newanta *et al.,* 2006). In the market surveys, a higher prevalence of NDV genome was detected during period 2 (dry season), which coincided with the Ethiopian Easter holiday in April, than during period 3 (wet season). In a survey targeting virus isolation at markets (Chapter 5), we also found more sick chickens and correspondingly more virus isolates, from sampling done during the dry season. This might be associated with poultry movements related with the Ethiopian Easter holiday that could facilitate disease spread. Orajaka *et al.* (1999) also noted more intense NDV activity during the cold dry period (Harmattan) in Nigeria, possibly

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associated with cold and windy weather or market-related poultry movements. Nevertheless, the proportion of virus genome detection might not necessarily indicate the actual infection status of household chickens as NDV neutralized by antibodies, i.e. non-infectious genome, may be detected by RT-PCR for a relatively long period after infection (Westbury *et al.,* 1984; Gohm *et al*., 2000; Fuller *et al*., 2010).

The lack of detectable antibodies to NDV or virus genome was noted in a high proportion of household flocks during wet (72.0%) and dry (57.6%) seasons, indicating that significant numbers of the village chickens are highly susceptible should virulent NDV infection occur (Allan and Gough,1974), as ND is highly transmissible in such flocks (van Boven *et al*., 2008). Henning *et al.* (2008) observed higher mortality rates in villages that had low proportions of birds with protective serological titres in Myanmar.

This study indicated the possible presence of class I viruses in backyard chickens of Ethiopia. This class is regarded as avirulent in chickens and has been recovered from waterfowl and shorebirds and also frequently from live bird markets elsewhere (Kim *et al.,* 2007a). The role of this class of virus in the epidemiology of ND is not clear for the moment and needs to be assessed. The presence of mild virus strains may contribute silently to increased antibody titres without visible clinical signs in infected chickens (Kite *et al.,* 2007).

In the partial F gene (239 bp) sequence analysis and sequence alignment the virus from markets and village samples grouped in similar clusters, which suggests that the same virus strains were circulating between markets and villages. However, this needs further investigation. During the study we also investigated an outbreak of disease in wild doves (*Streptopelia* spp.) in a village setting, which was diagnosed using a molecular test as caused by virulent pigeon PMV-1 (Chapter 5).

In conclusion, sero-epidemiology coupled with molecular analysis can be a useful tool to assess the status of NDV infection in an area and provide insights for further investigations. Our survey results showed that the village household chicken population is endemically infected with NDV, with a high proportion of household flocks experiencing new infections. Several circulating virus strains are believed to be the causes for the periodic outbreaks of the disease, with a tendency towards higher incidence during the dry season. This may also hold true for many other parts of Africa, or elsewhere in the world, where similar agro-ecological conditions exist. The presence of such virulent strains of NDV in household chickens could

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pose a significant threat to the development of the emerging small and medium scale commercial poultry production sector in Ethiopia. To reduce the seasonal mortality of the disease, vaccination at household level could be considered with due attention to its sustainability. In addition, further study is warranted to better understand the epidemiology of the disease and to characterize virus strains circulating in the study area, including the class I viruses, in order to properly aid control of ND.

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CHAPTER FOUR

HOUSEHOLD LEVEL RISK FACTORS FOR NEWCASTLE DISEASE SEROPOSITIVITY AND INCIDENCE OF NEWCASTLE DISEASE VIRUS EXPOSURE IN BACKYARD CHICKEN FLOCKS IN EASTERN SHEWA ZONE, ETHIOPIA

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4.1 Abstract

A cross-sectional study with repeated sampling was conducted to investigate potential risk factors for Newcastle disease (ND) seropositivity and for incidence of ND virus (NDV) exposure in household flocks of backyard chickens in Eastern Shewa zone, Ethiopia. Data were collected from 260 randomly selected households in 52 villages in Adami Tulu Jido Kombolcha and Ada'a woredas (districts) using a structured questionnaire, and serum samples from chickens were tested for NDV antibodies using a blocking enzyme-linked immunosorbent assay (ELISA). Sampling took place during September 2009 and the same households were again sampled in May 2010. Household-level seroprevalence and incidence of NDV exposure were estimated in various ways using serological results from the two samplings, flock dynamics, and farmers' reports of ND in their flocks. The risk factors were assessed using multivariable mixed-effects logistic regression models. Household-level seroprevalence at the two sampling times was 17.4% and 27.4%, respectively, and the estimated incidence of household-level NDV exposure during the intervening period ranged between 19.7% and 25.5%. At the first sampling, reduced frequency of cleaning of poultry waste was associated with increased odds of seropositivity (OR = 4.78; 95% CI: 1.42, 16.11; $P = 0.01$) while hatching at home vs. other sources (buying in replacement birds or receiving as gift) was associated with lower odds of seropositivity, both at the first sampling (OR = 0.30; 95% CI: 0.11, 0.82; $P = 0.02$) and the second sampling (OR = 0.23; 95% CI: 0.10, 0.52; $P < 0.001$). The risk of NDV exposure was shown to be higher with larger flock size at the beginning of the observation period (OR = 3.6; 95% CI: 1.25, 10.39; $P = 0.02$). Using an open water source (pond or river) for poultry compared to closed sources (tap or borehole) was associated with increased risk of NDV exposure (OR = 3.14; 95% CI: 1.12, 8.8; $P = 0.03$). The use of a grain supplement (OR = 0.14; 95% CI: 0.03, 0.69; $P = 0.03$) and hatching at home for flock replacement (OR = 0.23; 95% CI: 0.10, 0.52; $P = 0.005$) were associated with a lower risk of NDV exposure. Newcastle disease seroprevalence and incidence of NDV exposure were more heterogeneous between villages than between kebeles (aggregations of villages) and woredas in the study area. Further investigation of village-level risk factors would likely improve our understanding of ND epidemiology in backyard chickens.

Keywords: Backyard chickens, Ethiopia, Incidence, Newcastle disease, Risk factors, Seroprevalence

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4.2 Introduction

Backyard indigenous poultry are an important constituent of Ethiopian's poultry production system, constituting over 97% of the country's 49.3 million chickens (CSA, 2011). Most household flocks are small and of mixed age and feed mainly by scavenging. Chickens from different households may mix, potentially exposing them to disease-causing agents such as Newcastle disease virus (NDV) (Moges *et al*., 2010).

Newcastle disease (ND) is a viral infection caused by avian paramyxovirus serotype 1 (APMV-1), a single-stranded RNA virus with non-segmented genome that belongs to the genus *Avulavirus* of the family *Paramyxoviridae* (Mayo, 2002). It affects both young and adult chickens, causing severe economic and production losses worldwide and is regarded as the principal factor limiting rural village poultry production and extension (Awan *et al*., 1994; Alders and Spradbrow, 2001). This situation is no different in Ethiopia. Although there is no detailed study documenting mortality due to ND in Ethiopia, Nasser (1998) reported 50% mortality in village chickens around Addis Ababa, Debre-Zeit and Nazreth. Halima *et al.* (2007), in their study of village chickens in north-west Ethiopia, identified seasonal outbreaks of ND as the major cause of death. However, the epidemiology of the disease at household level, including factors that determine risk or facilitate spread, is poorly understood.

In traditionally managed village poultry the epidemic occurrence of ND depends on, in addition to presence of a pathogenic strain of NDV (Martin, 1992), a combination of factors such as age structure and immunity of the village flocks, concurrent infections and environmental factors which facilitate the spread of virus and affect the susceptibility of the birds (Awan *et al*., 1994). The major mode of transmission appears to be by the faecal-oral route in free range scavenging poultry, with the respiratory route playing a role where there are close bird-to-bird contacts (Martin, 1992). The main means by which virus can spread are movement of live birds, movement of people and equipment, movement of poultry products and contaminated poultry feed or water (Alexander *et al*., 2004). A report from Uganda related the disease risk with sales of chicken and increased socio-cultural activities among flock owners (Otim *et al*., 2007).

There is no preventive and control strategy undertaken against the disease in scavenging backyard poultry in Ethiopia. Knowledge of specific risk factors is a prerequisite to

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formulating appropriate control strategies; however, there is limited information on risk factors influencing the spread of the disease in village chickens in Ethiopia. Elsewhere, some of the risk factors reported for disease incidence, spread and persistence of infection among village chickens were the presence of latently infected carrier chickens, village poultry dynamics (selling, buying, giving), absence of preventive measures, unrestricted contact between village flocks and other poultry species including wild birds, disposal methods for poultry waste and carcasses, and re-stocking practices (Awan *et al*., 1994; Spradbrow, 1999; Otim *et al.,* 2007; Njagi *et al*., 2010; Rasamoelina Andriamanivo *et al*., 2012). Improved understanding of the risk factors for ND in backyard poultry production systems would help to prevent disease spread and formulate appropriate control strategies through better understanding of its epidemiology. The objective of this study was to identify potential household-level risk factors that are associated with either ND seropositivity or incidence of NDV exposure in backyard chicken flocks in the Eastern Shewa zone in central Ethiopia.

4.3 Materials and methods

4.3.1 Study areas

Ethiopia is divided into regions and then into zones and woredas (districts). Each woreda is composed of kebeles (district sub-units) which are aggregations of garees (villages), in turn made up of individual households. The studies were conducted in Adami Tulu Jido Kombolcha (ATJK) and Ada'a woredas of the Eastern Shewa zone of the Oromia region, Ethiopia. This study area was specifically selected for its poultry and human population density and the presence of wetlands, where other complementary studies were also undertaken. The climate of the area is temperate and sub-humid with a mean annual rainfall of 750-1100 mm, and average minimum and maximum temperatures of 8 and 35°C, respectively. It covers areas in the mid Rift Valley at an altitude of 1500-1900 m above sea level. The type of agriculture in the study area is small-scale crop and livestock farming.

4.3.2 Study design and data collection

A cross-sectional study, with two sequential data collection periods, was carried out in randomly selected households during September 2009 (wet season) and May 2010 (dry season) (Chapter 3). Broadly, the wet season in the study area corresponds to the months of

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June to September, sometimes extending to mid-October, while the dry season lasts from October to May. The households were selected using a multistage sampling design, and four chickens were sampled from each household. The unit of interest in this study was the household flock, which was defined as a group of chickens owned by an individual farmer. Households were randomly selected, provided at least four chickens were present and there was no history of any vaccination in the flock. The same households were sampled during both wet and dry seasons.

To calculate the required number of households to be sampled in order to estimate the prevalence of seropositive households, an expected prevalence (P_{exp}) of 35% (Biswas *et al*., 2008), desired absolute precision (d) of 10% and a confidence level of 95% were applied using the formula $n = 1.96^2 P_{exp}(1 - P_{exp})/d^2$ (Thrusfield, 2005) to give a required sample size of 88 households. However, multistage cluster sampling was used because of its practical advantages and flexibility. Therefore, the design effect (D) of the survey was calculated using the formula $D = 1 + (b - 1)roh$ (Bennett *et al*., 1991), where b is the number of samples per cluster and *roh* is the rate of homogeneity, equivalent to the intra-cluster correlation coefficient (ρ) in single-stage cluster sampling. In order to ensure the representation of a large number of villages, it was decided to sample only five households per village ($b = 5$). An intra-cluster correlation coefficient of $\rho = 0.18$ was reported for clustering of ND-positive birds within flocks (Otte and Gumm, 1997); however, in order to account for the multistage design and the unknown effect of clustering at higher levels, a much higher value of 0.5 was used for *roh*. Using the formula of Bennett *et al*. (1991), the design effect was therefore calculated to be $D = 3$ which, multiplied by the original calculated sample size, gave a required sample size of 264 households.

The list of kebeles (district sub-units) in each of the two study woredas (districts) was obtained from woreda agricultural offices and two kebeles were randomly selected from each. In each selected kebele a list of garees (villages) and households was compiled with the help of the local agricultural development agents; then 12-14 garees from each kebele, and finally five households from each selected garee, were randomly sampled depending on resource availability. In each selected household four chickens were selected and bled, and serum was harvested. Detail of the random selection processes was provided in Chapter 3.

In each household a pre-tested and standardized questionnaire (Appendix A) was used by the principal investigator in a face-to-face interview to collect information using the local

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language, Afaan Oromo. The questionnaire was administered immediately after bleeding of the chickens during each sampling period. Questionnaires for poultry owners investigated flock husbandry/management, flock structure and size, marketing practices and other potential risk factors (list shown in Table 4.2), as well as causes of chicken losses and farmers' perceptions about the occurrence of ND in their flocks (based on clinical signs suggestive of ND: including mass mortality, respiratory distress, diarrhoea, ocular and nasal discharges, or nervous signs). Questions related to conditions prevailing during the previous 8-12 months, or since the first sampling visit.

4.3.3 Serological testing

Serum samples were analysed (Chapter 3) with a commercially available blocking enzyme-linked immunosorbent assay (ELISA) (Svanovir NDV-Ab, SVANOVA Biotech, Uppsala, Sweden) using a NDV-specific monoclonal antibody (Czifra *et al*., 1996). The sensitivity and specificity of the test were reported to be 98.9% and 99.5%, respectively (Gohm *et al.,* 1999). A household flock was categorized as seropositive if one or more of the birds sampled showed detectable antibodies against NDV with a percentage inhibition (PI) value >40 according the manufacturer's.

4.3.4 Data analysis

Data were managed in a relational database (Microsoft Access), then transferred to a spreadsheet (Microsoft Excel) and analysed with STATA 11.1 (Stata Corp, College Station, TX, U.S.A.). Each of the following four definitions of ND seroprevalence (Chapter 3) or incidence of NDV exposure in a household was used:

1. Prevalence 1: Household seropositive (one or more seropositive birds present) at the first sampling, during the wet season.
2. Prevalence 2: Household seropositive at the second sampling, during the dry season.
3. Incidence A: Household seronegative at the first sampling AND seropositive at the second sampling AND with no additions to the breeding flock during the intervening period (to exclude the possibility of seropositive birds being introduced). Households seropositive at the first sampling were excluded from the population at risk.

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4. Incidence B: Household with an increase in the number of seropositive birds between the two samplings OR with a decrease in flock size combined with the farmer reporting ND in the flock.

Since the flocks under investigation were not vaccinated, seroconversion was used as a proxy for NDV exposure or infection at household flock level, along with other information as detailed above. The seroprevalence and incidence of NDV exposure in household flocks were calculated based on each of the above definitions, adjusting for the sampling weight (Chapter 3) for each household using the '*svy*' commands in Stata. Finally, each of the above outcomes was modelled as a function of the recorded risk factors, initially at the univariable level and then using multilevel logistic regression models (see below).

The following factors (explanatory variables) were considered in the risk factor analysis: flock size, night housing methods, cleaning frequency for poultry waste, flock restocking practice, poultry waste disposal practice, water source for chickens, purchase history, introduction of chickens from outside (including unsold chickens returning from market and gifts), presence of dogs in the household, contact with neighbouring chickens, distance to the next household keeping chickens (estimated by the interviewer), dead bird disposal practice and grain supplementation as a feed source besides scavenging. Household flock size was divided into terciles and modelled as a categorical variable. Distance to the next household was dichotomized using the median as cut-off. In the initial univariable analysis, the factors were first tested individually for their unconditional association with the outcome variables using a two-tailed Fisher's exact test. Those factors that were found to be associated with the outcome with $P < 0.25$ were considered for inclusion in the multilevel (mixed-effects) logistic regression models. However, the variable 'dead bird disposal' was excluded from the multivariable analysis because it applied only to those households in which chicken deaths occurred. A separate multivariable model was developed for each of the four outcome variables. Before inclusion, correlation or association between selected independent variables was investigated using pairwise correlation and cross tabulation of variables. The logistic models were reduced by backward elimination, removing each independent variable with $P_{Wald} > 0.05$ until all the remaining variables were significant ($P_{Wald} < 0.05$). All independent variables, including those not initially significant on univariable analysis, were then individually re-tested in the models and retained if significant. To account for the hierarchical nature of the data and clustering at the various levels, woreda was modelled as a fixed effect

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while kebele and garee were modelled as nested random effects. The results were reported as odds ratios with 95% confidence intervals and *P*-values. Models with and without random effects were compared using a likelihood-ratio test.

4.4. Results

4.4.1 Descriptive statistics

Serological and questionnaire data were obtained from 260 households in 52 villages during the first sampling period. Flock size (excluding small chicks of less than 2 months) ranged from 4 to 28 (median: 7; interquartile range (IQR): 5−10). At the second sampling, excluding households that had no chickens, flock size ranged from 1 to 36 (median: 6; IQR: 4−10). Questionnaire data were again obtained from the same 260 households; however eleven of them (4.2%) no longer had any chickens. In the remaining 249 households, 134 (53.8%) experienced flock reduction, of which 96 (71.6%) reported loss due to disease. Detailed flock dynamics is provided in Chapter 3. All surveyed households reported that their chickens had never been vaccinated against any poultry diseases and, as far as we could determine, no vaccination had been practised by any other households in the selected villages.

Table 4.1 shows the household-level NDV antibody seroprevalence obtained at the two sampling times (Chapter 3), and the two estimates of NDV exposure (incidence) during the intervening period, adjusted for sampling weights and clustering in the multistage design. Household-level seroprevalence varied from 17.4% in September 2009 to 27.4% in May 2010. Household-level incidence (exposure to NDV) estimates ranged from 19.7% based only on the subpopulation of 173 households seronegative at the first sampling, to 25.5% when information on within-flock seroprevalence, flock dynamics and farmers' reports of ND were incorporated.

4.4.1 Univariable analysis

Tables 4.2 and 4.3 show the unconditional associations of the categorical predictor variables with the four outcomes of interest. To avoid problems of collinearity with the variable 'introduction of chickens' (that included purchases, gifts or unsold returns from market), the variable 'bought chickens' (households that reported to have bought chickens) was excluded from the multivariable logistic regression. The variables selected for the multivariable models are indicated in Tables 4.2 and 4.3 using superscripts.

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Table 4.1 Estimates of household-level Newcastle disease seroprevalence and of incidence of Newcastle disease virus exposure in backyard poultry in Eastern Shewa zone, Ethiopia between September 2009 and May 2010.

Estimated parameters	Description	PAR[a]	Prevalence/ incidence (%)	95% CI
Prevalence 1	Proportion of households seropositive in September 2009	260	17.4	7.8, 34.2
Prevalence 2	Proportion of households seropositive in May 2010	249	27.4	14.3, 46.1
Incidence A	Proportion of seronegative households that became seropositive and did not introduce chickens	173	19.7	9.0, 37.9
Incidence B	Proportion of households with increase in number of seropositive birds or decrease in flock size with owner report of ND	260	25.5	18.3, 34.4

[a]population at risk (number of household flocks)

Table 4.2 Univariable analysis of household-level risk factors for Newcastle disease seropositivity among backyard chicken flocks in Eastern Shewa zone, Ethiopia in September 2009 and May 2010.

Variable	Level/category	Prevalence 1 (September 2009)			Prevalence 2 (May 2010)		
		n[c]	% pos[d]	*P*[e]	n[c]	% pos[d]	*P*[e]
Woreda	ATJK	130	16.15	0.626	127	22.05	0.035
	Ada'a	130	19.23		122	34.43	
Flock size [b]	<7	105	18.10	0.674	81	39.51	0.006
	7-9	81	14.81		82	28.08	
	>9	74	20.27		86	17.44	
Housing during night[ab]	Kitchen/store	56	21.43	0.123	52	36.54	0.059
	Main house with people	99	11.11		100	19.00	
	Separate chicken house	52	25.00		53	32.08	
	Outside (under roof, on trees)	53	18.87		44	34.09	

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Cleaning frequency[ab]	Daily	161	13.04	0.011	152	23.68	0.075
	Weekly	31	35.48		36	27.78	
	<1/week or never	68	20.59		61	39.34	
Poultry waste disposal	Thrown nearby	201	16.92	0.563	181	26.52	0.429
	Thrown far away	59	20.34		68	32.35	
Water source for	Closed (tap or borehole)	102	16.67	0.026	100	19.00	0.024
chickens[ab]	Partly closed (hand-dug wells)	85	25.88		83	36.14	
	Open (pond or river)	73	9.59		66	31.82	
Flock restocking sources[ab]	Other sources	34	35.29	0.007	49	53.06	<0.001
	Hatching at home only	226	15.04		200	22.00	
Bought chickens	Yes	55	29.09	0.017	57	40.35	0.028
	No	205	14.63		192	24.48	
Introduction of chickens	Yes	59	27.12	0.051	64	37.50	0.075
from outside[ab]	No	201	14.93		185	24.86	
Contact with neighbouring	Yes	229	18.78	0.127	230	28.70	0.600
chickens[a]	No	29	6.90		19	21.05	
Distance to neighbouring	≤ 30m	145	20.69	0.191	138	32.61	0.090
household [ab]	>30 m	115	13.91		111	22.52	
Own dog[a]	Yes	178	15.73	0.226	177	26.55	0.438
	No	82	21.95		72	31.94	
Grain supplement	Yes	248	18.15	0.690	240	27.08	0.120
provided[b]	No	12	8.33		9	55.56	
Selling of chickens/eggs[b]	Yes	238	17.65	1.000	226	39.13	0.229
	No	22	18.18		23	26.99	
Dead bird disposal	Thrown nearby	70	21.43	0.090	69	23.19	0.276
	Thrown far away	93	23.66		112	33.04	
	Burn or bury	14	50.0		16	37.5	

[a] variable significant with P<0.25 and selected for multivariable logistic regression model (Prevalence 1).

[b] variable significant with P<0.25 and selected for multivariable logistic regression model (Prevalence 2).

[c] Number of household flocks. [d] Household-level ND seropositivity. [e] Fisher's exact test *P*-value.

Table 4.3 Univariable analysis of risk factors for incidence of Newcastle disease virus exposure among backyard chicken flocks in Eastern Shewa zone, Ethiopia between September 2009 and May 2010.

Variables	Level/category	Incidence A			Incidence B		
		n[c]	% pos[d]	*P*[e]	n[c]	% pos[d]	*P*[e]
Woreda	ATJK	91	17.58	0.566	130	20.77	0.088
	Ada'a	82	21.95		130	30.77	

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Flock size [a,b,f]	<7	68	10.29	0.032	105	20.00	0.212
	7-9	51	23.53		81	29.63	
	>9	54	27.78		74	29.73	
Housing during night[a]	Kitchen/store	35	31.43	0.163	55	29.09	0.318
	Main house with people	73	13.7		102	19.61	
	Separate chicken house	35	22.86		54	29.63	
	Outside (under roof, on trees)	30	16.67		49	30.61	
Cleaning frequency[b]	Daily	106	19.81	0.962	156	21.79	0.029
	Weekly	24	16.67		36	19.44	
	<1/week or never	43	20.93		68	38.24	
Poultry waste disposal	Thrown near by	131	18.32	0.504	191	26.18	0.870
	Thrown far away	42	23.81		69	24.64	
Water source for	Closed (tap or borehole)	71	12.68	0.034	102	18.63	0.076
chickens[ab]	Partly closed (hand dug wells)	52	17.31		89	28.09	
	Open (pond or river)	50	32.0		69	33.33	
Flock restocking sources[b]	Other sources	3	33.33	0.484	52	42.31	0.004
	Hatching at home only	170	19.41		208	21.63	
Bought chickens	Yes	8	25.00	0.686	59	21.63	0.028
	No	165	19.39		201	22.39	
Introduction of chickens	Yes	15	18.75	1.000	66	34.85	0.072
from outside[b]	No	158	19.62		194	22.68	
Contact with neighbouring	Yes	156	21.15	0.200	239	26.78	0.299
chickens[a]	No	17	5.88		21	14.29	
Distance to neighbouring	≤ 30m	87	21.84	0.567	145	28.97	0.201
household[b]	>30 m	86	17.44		115	21.74	
Own dog	Yes	126	22.92	0.525	183	24.04	0.353
	No	48	22.92		77	29.87	
Selling of chickens/eggs[b]	Yes	161	19.88	1.000	235	23.83	0.051
	No	12	16.67		25	44.00	
Grain supplement	Yes	167	19.16	0.336	250	24.4	0.021
provided[b]	No	6	33.33		10	60.0	
Dead bird disposal	Thrown near by	53	20.75	0.950	77	25.97	0.709
	Thrown far away	72	23.61		114	31.58	
	Burn/bury	11	18		16	31.28	

[a]variable significant with P<0.25 and selected for multivariable logistic regression model (Incidence A)

[b]variable significant with P<0.25 and selected for multivariable logistic regression model (Incidence B)

[c] Number of household flocks. [d] Household-level ND seropositivity. [e] Fisher's exact test *P*-value.

[f] Flock size at the beginning of the observation period (September 2009).

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4.4.2 Multilevel mixed-effects logistic regression models

The final mixed-effects logistic regression models are presented in Table 4.4 for ND seroprevalence and Table 4.5 for NDV exposure (incidence). Risk factors differed somewhat between the four outcomes.

Table 4.4 Final multivariable mixed-effects logistic regression models of risk factors for Newcastle disease virus seropositivity among backyard chicken flocks in Eastern Shewa zone, Ethiopia in September 2009 and May 2010.

Variable and level	Outcome					
	Prevalence 1[a] (September 2009)			Prevalence 2[b] (May 2010)		
	OR	95% CI (OR)	*P*	OR	95% CI(OR)	*P*
Woreda						
ATJK	1	-	-	1	-	-
Ada'a	0.57	0.17;1.78	0.344	2.09	0.84;5.18	0.110
Flock size						
<7				1	-	-
7-9				0.67	0.29;1.52	0.336
>9				0.28	0.11;0.67	0.004
Cleaning frequency						
Daily	1	-	-			
Weekly	4.78	1.42; 16.11	0.012			
<1/week or never	2.28	0.77;6.8	0.138			
Flock restocking source						
Other sources	1	-	-	1	-	-
Hatching at home only	0.30	0.11;0.82	0.020	0.23	0.10;0.52	<0.001
Random effects: Variance (SE)						
Kebele	2.68×10^{-18} (3.15×10^{-9})			1.74×10^{-20} (6.9×10^{-11})		
Garee	1.26 (0.77)			1.17 (0.66)		

[a]Prevalence 1 model: Likelihood ratio test vs. standard logistic regression model: $P = 0.013$

[b]Prevalence 2 model: Likelihood ratio test vs. standard logistic regression model: $P = 0.007$

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Table 4.5 Final multivariable mixed-effects logistic regression models of risk factors for incidence of Newcastle disease virus exposure among backyard chicken flocks in Eastern Shewa zone, Ethiopia between September 2009 and May 2010.

Variable and level	Outcome					
	Incidence A[a]			Incidence B[b]		
	OR	95% CI (OR)	*P*	OR	95% CI(OR)	*P*
Woreda						
ATJK	1	-	-	1	-	-
Ada'a	1.36	0.53;3.49	0.527	1.55	0.64;3.76	0.336
Flock size [c]						
<7	1	-	-			
7-9	2.74	0.93;8.09	0.067			
>9	3.6	1.25;10.39	0.018			
Water source for poultry						
Closed (tap, borehole)	1	-	-			
Partly closed (hand-dug	1.25	0.37;4.12	0.716			
wells)	3.14	1.12;8.8	0.029			
Open (pond, river)						
Flock restocking source						
Other sources				1	-	-
Hatching at home only				0.32	0.15;0.70	0.005
Grain supplement						
No				1	-	-
Yes				0.16	0.03;0.69	0.025
Random effects: Variance (SE)						
Kebele	$1.79x10^{-17}$ ($5.6x10^{-9}$)			$7.12x10^{-12}$ (0.248)		
Garee	0.35 (0.06)			1.07 (0.291)		

[a]Incidence A model: Likelihood ratio test vs. standard logistic regression model: $P = 0.419$.

[b]Incidence B model: Likelihood ratio test vs. standard logistic regression model: $P = 0.005$.

[c] Flock size at the beginning of the risk period.

In the first sampling period (September 2009), increased odds of ND seropositivity was associated with decreased poultry waste cleaning frequency (OR = 4.78; 95% CI: 1.42; 16.11) whilst reduced odds of seropositivity was associated with using hatching at home as flock rebuilding strategy compared with obtaining birds from other sources (OR = 0.30; 95%

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CI: 0.11, 0.82) (Table 4.4). Contact with neighbouring chickens tended to increase the odds of seropositivity (OR = 4.69; 95% CI: 0.85, 25.78), although this was not significant (*P* = 0.06) and was dropped from the model. At the second sampling (May 2010) a reduced odds of seropositivity was significantly associated with hatching at home for stock rebuilding (OR = 0.23; 95% CI: 0.1, 0.52). The model revealed also that the odds of seropositivity was 3.6 times greater in flocks of <7 chickens compared to flocks with >9 chickens (Table 4.4). The random effects parameters showed that there was clustering at village level, i.e. household seropositivity varied between villages, while there was very little variation between kebeles.

In the first incidence model (Incidence A) the variables found associated with the increased risk for new NDV exposure/infection in a household flock, were larger flock size at the beginning of the risk period (OR = 3.6; 95% CI: 1.25, 10.39) and an open water source for chickens compared to closed (tap or borehole) or partly closed (hand-dug well) water sources (OR = 3.14; 95% CI: 1.12, 8.8) (Table 4.5). In the second incidence model (Incidence B), using hatching at home for stock rebuilding was found to be a protective factor (OR = 0.32; 95% CI: 0.15, 0.7) for NDV infection in the household flock. Similarly, feeding of a grain supplement besides scavenging appeared to reduce risk of NDV exposure (OR = 0.16 95% CI: 0.03; 0.69) (Table 4.5). The variance component showed that there was clustering at village level, i.e. household seropositivity varied between villages, while for kebeles the variance was not significantly different from zero.

4.5 Discussion

The study investigated household-level risk factors for NDV infection in household chicken flocks based on a cross-sectional serological survey with two sampling periods 8 months apart. In natural infections, HI antibodies decline after 3 to 4 months and have disappeared by 8 to 12 months post-infection (Martin, 1992). In longitudinal monitoring of village chicken flocks Serrão *et al*. (2012) noted that antibodies rose and declined over a period of 3-6 months. Therefore, in the absence of re-exposure or vaccination, antibody titres gradually decline to become undetectable and seropositivity can be considered a proxy for recent exposure to NDV. Our first estimate of the incidence of NDV exposure (19.7%) is therefore likely to be conservative, given the short duration of titres, particularly as it only included cases in which we were certain that seroconversion, i.e. exposure, had taken place in the

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actual flock. In addition, we may have biased the estimate downward by excluding households that bought in chickens, which were shown to be more at risk in the other model. Indeed, without excluding households that bought in chickens, 24% (49/204) of flocks that were seronegative at the first sampling became seropositive. Flocks which were seropositive at both samplings likely do not indicate seroconversion carried over from the first survey, given the short duration of titres; in fact, 90% (19/21) of the households that were seropositive at both samplings reported clinical ND. In addition, 11 households (ten of which were seronegative during first survey) lost their entire flock mainly due to disease conditions during the observation period. All of this suggests that our first incidence estimate was an underestimate of the true incidence of NDV exposure. Our second approach (Incidence B) also accounted for households that were initially seropositive but where further viral circulation occurred, and additionally incorporated information from farmers regarding likely occurrence of ND in their flocks during the study period. Newcastle disease, especially in its virulent form, is well recognized by the farmers in our study area and therefore we considered it valuable information to include in the definition of incidence. Therefore, we believe that the second incidence estimate (25.5%) was likely closer to the true proportion of households in which NDV circulation occurred. It may have included some false positives (lack specificity) because of farmers' misdiagnosis; on the other hand, it may also have missed some households in which seroconversion occurred but titres declined within the 8 month period, or where mortalities occurred and the farmer restocked.

The odds of seropositivity in the second survey were significantly higher in small flocks of less than 7 chickens. This could be explained by the fact that velogenic ND resulted in mortalities, reducing the flock size. Flocks that had experienced ND were therefore likely to be smaller than those that had not, *i.e.* small flock size is likely an outcome of ND rather than a risk factor. In fact, incidence of the NDV exposure was found to be higher in flocks with larger size at the beginning of the observation period compared to smaller ones (Table 4.5). Larger flocks are more likely to contain chickens of various ages, with varying susceptibility to ND, hence increasing the risk of NDV transmission within the flock. Also, the larger the flock, the greater the likelihood that one or more birds will come into contact with outside sources of infection, e.g. wild birds, or through scavenging.

In all the models except the third (incidence A), in which households that purchased chickens were specifically excluded, using only hatching at home as flock rebuilding strategy was

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protective compared to using other sources (purchasing from local market and receiving as a gift). The fact that the variable 'introduction of chickens' was marginally significant in the univariable analyses but not in the multivariable models was likely due to its correlation with purchase of chickens for restocking. Markets could be potential sources for NDV infection (Awan *et al*., 1994) or already seropositive chickens (Chaka *et al*., 2012a). Restocking using birds from markets often occurs either after disease outbreaks that resulted in total loss of chickens or after flocks has been intentionally downsized during wet seasons and crop farming seasons. There could be a chance of purchasing clinically normal chickens that are infected with virulent virus (Fantanilla *et al*., 1994). Otim *et al*. (2007) also identified that restocking using chickens from markets was a potential risk factor for ND outbreaks in village chickens in Uganda. A similar observation was noted with live chickens from markets in Vietnam (Nguyen, 1992). In Madagascar it was also reported that farms using many small markets were at greater risk of ND (Rasamoelina Andriamanivo *et al*., 2012). The finding that households maintaining a closed flock are at lower risk of NDV exposure is therefore consistent with previous reports, and suggests that this may be a useful preventive measure.

It was apparent that using open water sources increased the risk of incidence of ND in household flocks, more specifically in households that used ponds and rivers. Such water sources, unlike tap or borehole water, can easily be contaminated by faecal droppings from wild birds or other poultry, which could be carriers of NDV. It was reported that NDV has been isolated from about 240 different bird species (Kaleta and Baldouf, 1988), and velogenic virus has been recovered from apparently healthy pigeons and chickens in Nigeria (Echeonwu *et al*., 1993). Water contaminated with faeces from infected birds may introduce NDV to a flock (Awan *et al*., 1994; Alexander, 1995; Brown *et al*., 2007), and this is likely also to have been the case in our study area.

Although not included in the final multivariable model, contact with neighbouring chickens tended to increase the odds of seropositivity at the first sampling (OR = 4.79, 95% CI: 0.90, 25.37). The typical scavenging behaviour of village chickens, moving from place to place, exposes them to frequent contacts allowing easy spread of the disease once it has started in one household flock, and explaining the significant clustering of seropositivity within villages. The feeding of a grain supplement was associated with lower incidence of NDV exposure. This may be due to its effect on chicken behaviour, since chickens fed with grains every morning may be more likely to stay near home rather than wander around to scavenge

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and hence come into contact with other chickens. In the univariable analysis there was a tendency for an inverse association between distance to neighbouring household and odds of seropositivity. However, this was not the case in the multivariable models, although it is logical to assume that the closer the distance the greater the chance for mixing of flocks from different households and transmission of disease. This suggests that there are other management, demographic and behavioural factors, apart from simply distance, which determine direct or indirect contacts between household flocks. One such factor could be waste and carcass disposal, which was done by most of the households in an open area, easily accessible to scavenging chickens. Njagi *et al*. (2010) reported a significant association of mode of disposal of dead birds and poultry wastes with ND outbreaks in village chickens in Kenya. In our study, however, because these practices would have an effect on risk of infection for other households in the village rather than the households practising them, this was not identified as a household-level risk factor. One important observation by the farmers was that movement of chickens between households situated far apart occurred through cocks, whereas hens usually did not move far from the main house to scavenge. Only 2.7% (7/260) of the households mentioned that they immediately confined their chickens during a suspected outbreak of ND in the village; this was not investigated further, however biosecurity in village chickens has been reported to be generally poor in Ethiopia (Wossene, 2006).

Type of housing did not appear to be a risk factor in this study, unlike in an intensive system where poultry are kept in close contact, facilitating transmission. It was thought that chickens that stayed outside or perched on trees would be less exposed to the disease, also considering the inability of sick birds to fly up into trees (Huchzermeyer, 1993). However, those chickens housed in human dwellings at night tended to have lower odds of seropositivity in the univariable analysis (Table 4.2). The fact that this was not significant in the multivariable model was likely due to confounding by cleaning frequency, since in the main house cleaning of poultry waste was almost always practised daily, reducing the chance of faecal-oral transmission. In the multivariable model (Prevalence 1), daily cleaning was a protective factor compared with weekly cleaning. It has been established that NDV can survive in poultry faeces for several days to weeks depending on temperatures (Kinde *et al*., 2004).

The presence of pets was not identified as a risk factor, although of the households which reported to have had ND outbreaks, 22% considered it to be associated with dogs bringing in

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dead birds from other places and chickens scavenging on them. However, as discussed for waste and carcass disposal above, the effect of movement of and contamination by dogs would likely not be limited to a single household, but have village level implications in disease spread; such factors require further investigation.

There are inherent difficulties in detecting NDV circulation and accurately estimating incidence using serological methods, particularly since the disease can be highly fatal and result in depopulation of the flock, or antibody titres may be short-lived. The fact that a household had no seropositive birds did not necessarily mean that the disease had never occurred. For example, in some households we were informed that incubating hens had survived a suspected ND outbreak and eventually tested negative during the survey. Some misclassification of the outcome is likely to have occurred, which may have reduced the power of our analyses to detect significant risk factors. In addition, the study area, although typical of the village poultry rearing system in much of Ethiopia, did not cover a wide variety of agro-ecological zones and other husbandry practices, where different risk factors may be important (Wilson, 2010).

In conclusion the study revealed that Newcastle disease is common in household flocks in our study area and is fairly evenly distributed across woredas and kebeles, but is significantly clustered within villages. The source for flock restocking, flock size, cleaning frequency, water sources, and feeding of a grain supplement were independently associated either with NDV seropositivity or with incidence of NDV exposure. Further investigation of factors which operate at village level rather than individual household level would likely improve our understanding of risk factors for ND in backyard chickens.

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CHAPTER FIVE

MOLECULAR CHARACTERIZATION AND PHYLOGENETIC ANALYSIS OF NEWCASTLE DISEASE VIRUS ISOLATED IN BACKYARD CHICKENS AT VILLAGES AND MARKETS IN ETHIOPIA

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5.1 Abstract

Newcastle disease (ND) is an economically important disease causing considerable losses around the world, more pronounced in developing countries like Ethiopia where village poultry are kept. No Ethiopian ND virus (NDV) strains have been completely characterized. In this study, samples were collected from suspected ND cases in village chickens at live bird markets, during village surveys or at veterinary clinics. Viruses were isolated in embryonated eggs, and characterized by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) coupled with fusion (F) and haemagglutinin-neuraminidase (HN) genome sequencing. The isolates gave positive results on F gene based rRT-PCR with the cleavage site motifs ^{112}RRRKRF^{117}, ^{112}RRQKRF^{117}, ^{112}RRHKRF^{117} and ^{112}KRRKRF^{117}, indicative of virulent strains. Phylogenetic analysis, based on sequencing of F (1047 bp) and HN (1713bp) genes, from five isolates classified these isolates into genotype VI, sharing a common ancestor with pigeon paramyxovirus-1 (PPMV-1). However, it clustered well apart from other PPMV-1 strains in the sub-genotype (VIb), to be considered a possible new sub-genotype. The phylogenetic proximity of the isolates to PPMV-1 suggested that viral transmission may occur between wild birds and poultry. Further investigation into this is required, as continuous surveillance in domestic and wild birds in a wider area of the country. The study showed the circulation of closely similar virus strains between markets and villages. The clustering of isolates from spatially distant areas suggests the possibility of spread of NDV through market-related live chicken movements.

Key words: Newcastle disease virus, backyard chickens, Ethiopia, phylogenetic analysis

5.2 Introduction

Newcastle disease (ND) is caused by avian paramyxovirus serotype 1 (APMV-1), which, with viruses of the other nine APMV serotypes (APMV-2 to APMV-10), have been placed in the genus *Avulavirus*, belonging to the sub-family *Paramyxovirinae*, family *Paramyxoviridae* (Mayo, 2002; OIE, 2008b; Miller *et al*., 2010a). Newcastle disease virus (NDV) is a single-stranded RNA virus with an envelope bearing spikes, containing the components that initiate haemagglutination (HA). Newcastle disease virus can cause clinical signs ranging from subclinical infections to 100% mortality, depending on the pathotype, but also on the host

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characteristics: species susceptibility, age, presence of co-infection, environmental stress and immune status (Martin, 1992).

Currently NDV have been classified according to two different systems: either in genetic lineages (Aldous *et al*., 2003) or in classes (Czeglédi *et al*., 2006). The system based on genetic lineages groups NDV into six lineages and 13 sublineages, with one additional lineage (lineage 7) and seven sub-lineages suggested later (Snoeck *et al*., 2009; Cattoli *et al*., 2010). In the second system based on classes, NDV strains are divided into two clades (class I and class II) (Czeglédi *et al*., 2006). Class I includes almost exclusively low virulent strains recovered from wild waterfowl worldwide. Class II is comprised of ten genotypes (I-X) that include strains of low and high virulence isolated from poultry and wild birds (Tasi *et al*., 2004; Miller *et al*., 2010b). An additional genotype (XI) was proposed recently from Madagascar (Maminiaina *et al*., 2010). The genotypes that are considered ‘‘early’’ (emerged before 1960), namely I, II, III, IV and IX contain genomes of 15,186 nucleotides in length (Czegledi *et al*., 2006), while viruses that emerged after 1960, namely V, VI, VII, VIII, and X contain genomes of 15,192 nucleotides in length. Genotypes I and II of class II consist mainly of low virulence viruses; and genotype II strains are commonly used for vaccine production. Genotypes V, VI, VII, and VIII contain only virulent viruses. Genotype VI is further divided into sub-genotypes VIa through VIg, with VIb being commonly isolated from pigeons (Miller *et al*., 2010b). Genotype VII represents viruses that emerged in the Far East and also spread to different part of Africa (Abolnik *et al*., 2004; Soneck *et al*., 2009; Cattoli *et al*., 2010; Hassan *et al*., 2010). Advanced studies in molecular testing and virus characterization enable researchers to continuously discover novel genotypes or lineages/sublineages. In view of the limitations of having two classification systems for NDV that generate confusion and discrepancies, Diel *et al.* (2012) have recently proposed a unified nomenclature and classification system for NDV that categorises the viruses into 15 genotypes.

The NDV genome is composed of six genes, encoding their corresponding structural proteins: nucleoprotein (NP), phosphoprotein (P), matrix (M), fusion (F), haemagglutinin-neuraminidase (HN), and the RNA polymerase (L) (Chambers *et al*., 1986; Yusoff and Tan, 2001). Phylogenetic studies of both the F protein and the HN protein genes of NDV have been used for molecular epidemiologic analysis and characterization (Ballagi-Pordany *et al.,* 1996; Ke *et al*., 2001; Otim *et al*., 2004) and to group NDV into specific lineages or genotype

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by estimating the average evolutionary divergence over sequence pairs (Westbury, 2001; Tamura *et al.,* 2004). Molecularly, the different pathotypes are characterized by amino acid sequence of the cleavage site of fusion protein (Aldous *et al.*, 2003), which was recognized as the major determinant in virulence (de Leeuw *et al*., 2005; OIE, 2008b). The presence of multiple basic amino acids located at the C-terminus of the F_1 protein and phenylalanine (F) at the N-terminus of the F_2 protein, which correspond to the cleavage site of the precursor F_0, is indicative of virulent virus while the low virulent NDV have fewer basic amino acids at this site (OIE, 2008b).

Molecular analysis, along with further characterisation of NDV field isolates, is useful in order to identify the type of strains circulating in a particular geographic area (Singh *et al*., 2005). In Africa molecular studies in various geographical areas and time periods have revealed the existence of several genotypes or lineages, including novel or new virulent strains (Abolink *et al*., 2004; Otim *et al*., 2004; Snoeck *et al*., 2009; Servan de Almeida *et al*., 2009; Cattoli *et al*., 2010; Yongolo *et al*., 2011; Van Borm *et al*., 2012). In Ethiopia ND was first described and isolated in 1971 from an outbreak in Eritrea, then part of Ethiopia (NVI, 1974; Bawke *et al*., 1991). Since then the disease has spread throughout the country, causing losses in indigenous village chickens. Sequence analysis, genetic pathotyping and detailed genetic characterization of NDV in rural poultry are of paramount importance to understand the circulating genetic lineage characteristics and to trace their epidemiological sources. However, the genetic characteristics of the virus strains circulating in Ethiopia are largely unknown and no information was available from the published literature. The objective of this study was to conduct molecular characterization of and to investigate epidemiological relationships between NDV isolates obtained from backyard chickens in villages and markets in the mid-Rift Valley area of central Ethiopia.

5.3. Materials and methods

5.3.1 Study area, sample collection and virus isolation

The study was conducted on the isolates obtained in villages and markets in the mid-Rift Valley area of Oromia region and neighbouring areas where other epidemiological studies were undertaken (Figure 5.1). Cloacal and tracheal swabs were collected from chickens in household flocks during village surveys, from clinically ill birds at live poultry markets during market surveys or from cases admitted to veterinary clinics between years 2010-2011.

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Swabs were placed in virus transport medium (VTM), kept on ice in the field and transported to the laboratory and stored at -80°C until processed. When possible, sick birds were purchased, euthanized, a necropsy performed and tissues from various organs were collected (lung, trachea, intestine, liver, spleen, brain). Viruses were isolated from cloacal and tracheal swab material or from tissue homogenates by standard virus isolation methods in embryonating chicken eggs (Alexander, 1998; OIE, 2008b). Briefly, nine to eleven-day-old embryonated chicken eggs were inoculated via the allantoic cavity with 0.2 mL of the aliquot or tissue homogenate (three eggs per sample) and incubated at 37°C. The eggs were candled daily for four days; those with visible embryo death were chilled to harvest allantoic fluid. At the end of the fourth day all eggs were chilled for allantoic fluid harvest.

5.3.2 Haemagglutination and haemagglutination inhibition assays

The haemagglutination (HA) and haemagglutination inhibition (HI) assays were completed by microtitre methods as described by the World Organisation for Animal Health (OIE) (OIE, 2008b) at the National Animal Health Diagnostic and Investigation Centre (NAHDIC), Sebeta, Ethiopia. Confirmation of HA positive fluids was done using the HI assay. Four HA units of the viral test antigen (in allantoic fluid) were used in the HI assay with NDV specific pathogen free standard antiserum obtained from Veterinary Laboratories Agency, Weybridge, UK (Lot 1/09). Sixty three allantoic fluids that tested positive on HA and HI were sent for further analysis to the *Centre de Coopération Internationale en Recherche Agronomique pour le Développement* (CIRAD), Montpellier, France.

5.3.3 Viral RNA extraction and molecular detection

Allantoic fluids positive on HA and HI were tested by real-time reverse transcriptase polymerase chain reaction (rRT-PCR) assay at CIRAD. Viral RNA was extracted from allantoic fluids by a high throughput automated workstation, Biomek FX^{P} (Beckman Brea, California, USA), using a Nucleospin RNA virus kit (Macherey Nagel Düren, Germany) according to manufacturer's instructions. The viral RNA was re-suspended in nuclease-free water and stored at -80°C. The F gene of NDV was detected by one step rRT-PCR (in-house protocol, CIRAD) using the Stratagene Mx3000 or 3005 (Foster , California, USA). The forward primer was F+259 5'- ACAYTGACYACTTTGCTCA_3' and the reverse primer was F488rev 5'- TGCACAGCYTCATTGGTTGC-3'. The primers were designed according to an alignment of 500 sequences of the F gene from GenBank data. RT-PCR was carried out

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in a 25 µl reaction mixture with the Brilliant SYBR Green rRT-PCR II Master Mix Kit (Stratagene) according to the manufacturer's instructions. The conditions for reverse transcription were 50°C for 30 min and 95°C for 15 min. PCR consisted of 40 cycles of 95°C for 30 sec, 60°C for 1 min, and 72°C for 30 sec, followed by the final dissociation stage: 1 min at 95°C, 30 sec at 55°C and 30 sec at 95°C. A serial dilution of a ND vaccine strain available at CIRAD and ultra-pure water were used as positive and negative controls, respectively.

5.3.4 Amplification for sequencing

Stock for sequence analysis was prepared by again inoculating rRT-PCR positive allantoic fluid samples into the allantoic cavities of 9-11 day old embryonated fowls' eggs originating from a commercial specific pathogen free farm to produce concentrated stock of virus for sequencing. Allantoic fluid from dead eggs were then stored at -80°C and used as a working stock for sequence analysis. Viral RNA was extracted from all ND positive samples and complementary DNA (cDNA) was synthesized with oligo dT primer using SuperScript III First-strand Synthesis SuperMix (Invitrogen) according to manufacturer's instructions. Conventional PCR was carried out on 20 ng of cDNA using Platinum Taq DNA Polymerase High Fidelity kit (Invitrogen) according to manufacturer's instructions. Initially, partial F gene (154 bp) sequencing, including the cleavage site, of all rRT-PCR positive isolates was undertaken and pathotype characteristics were identified. Four distinct PCR allowed the amplification of the complete sequence of the F and HN genes of five selected isolates to ensure a consensus sequence. The primers used for PCR amplification are listed in Table 5.1. The PCR products were sequenced by Beckman Coulter Company (http://www.beckmangenomics.com/) using the same primers described for PCR reactions and internal F and HN primers for assembling contigs (Table 5.1).

5.3.5 Phylogenetic analysis

The F and HN genes were sequenced to determine the phylogenetic relationships of the selected Ethiopian isolates (n = 5; purposely selected to represent the studied geographical area) to previously reported NDV sequences from GenBank pertaining to available genotypes (Czegledi *et al*., 2006). Sequences assembly and editing was performed in Vector NTI Advance™ 11 software (http://www.invitrogen.com/site/us/en/home/Products-and-Services/Applications/Cloning/vector-nti-software/vector-nti-advance-software.html).

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Table 5.1 Source of the primers used in amplification of the F and HN genes.

Primer	Reference	Primer sequence 5' - 3'	PCR Amplicon size	Sequencing
MFS1 (forward)	Fuller *et al*. (2007)	GACCGCTGACCACGAGGTTA	1299 bp (F gene)	F gene
11L (reverse)	Anses laboratory[a]	GGAATGTCACTATTCTTGTAC		
11 U (forward)	Anses laboratory	CTTAARGAGAGCATTGCTGCA	1600 bp (F gene)	F gene
13 L (reverse)	Anses laboratory	GAGATCRCAGTCGRTATGCC		
10 L	Anses laboratory	CCAACTGCCACTGCTAGTTG		F gene
12 L	Anses laboratory	GTTAAAACRATATARGTAATG AG		F gene
12 U	Anses laboratory	AAAGGRTTTGCCTCRGCACT		F gene
P6A (forward)	Zou *et al*. (2005)	ATCAGATGAGAGCCACTACA	1120 bp (HN	HN gene
HN886 (reverse)	CIRAD primer	ACTCCTGGGTAATTTGCCAC	gene)	HN gene
3HNOV (forward)	Fuller *et al*. (2007)	GTCTTGCAGTGTGAGTGCAAC	1271 bp (HN gene)	HN gene
P7B (reverse)	Zou *et al*. (2005)	TCTGCCCTTTCAGGACCGGA		HN gene

[a]Agence Nationale de Sécurité Sanitaire (http://www.anses.fr/)

Homology reference sequence selection was based on a BLAST search (Zhang *et al*., 2000) where viruses with complete gene sequences with clear indication of isolate identity, host and country of origin were included. Two isolates previously isolated from outbreak cases (ETH762_chicken_2007 and ETH303_dove_2006) were also included in the phylogenetic study. Multiple nucleotide sequence (DNA pair-wise) alignment, with the representative genotypes from GenBank, was done using MegAlign (ClustalW) in Mega software (version 4.0) (Tamura *et al*., 2007). Results from sequencing of partial F gene (1047 bp) and complete HN gene (1713 bp) on five isolates and reference sequence were used to construct a phylogenetic tree. Maximum-likelihood (ML) phylogenetic analysis with 1000 bootstrap replicates was performed using PhyML under the general time reversible (GTR) model in Treefinder (Jobb, 2011) (version of March 2011). Class I viruses (DQ097393) were used as an out-group. The previously described genotype-based nomenclature is used for identification of clades throughout this chapter (Czegledi *et al*., 2006). Results are presented as rooted neighbour joining trees with percentage values by bootstrap resampling of 1000 replicates. In order to test the reliability of the lineages defined for NDV, pair-wise sequence comparisons (PASC) were performed using 50 F gene sequences of NDV strains included in the tree from the GenBank. Mean distances among and within lineages were calculated using

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PASC in MEGA4 software. Analyses were conducted using the Tamura-Nei model (Tamura *et al*., 1993).

5.4. Results

5.4.1 Virus isolation and detection by rRT-PCR

In total 63 NDV isolates were obtained from 20 different sites (markets, villages or clinics) based on their positive HA and HI results. While a few samples were obtained from 6 dead birds the remaining were obtained from live or moribund birds. Figure 5.1 shows the geographic locations where the NDV isolates were obtained and the identification and epidemiological information of the isolates are shown in Table 5.2. Partial F gene (154 bp) sequencing, of the region spanning F_0, of all rRT-PCR positive isolates revealed the amino acid sequence motifs G/RRRKR/FV, G/RRQKR/FV, G/RRQKR/FA, G/RRHKR/FV and G/KRRKR/FV (Table 5.2), indicative of virulent strains (pathotype) (OIE, 2008b).

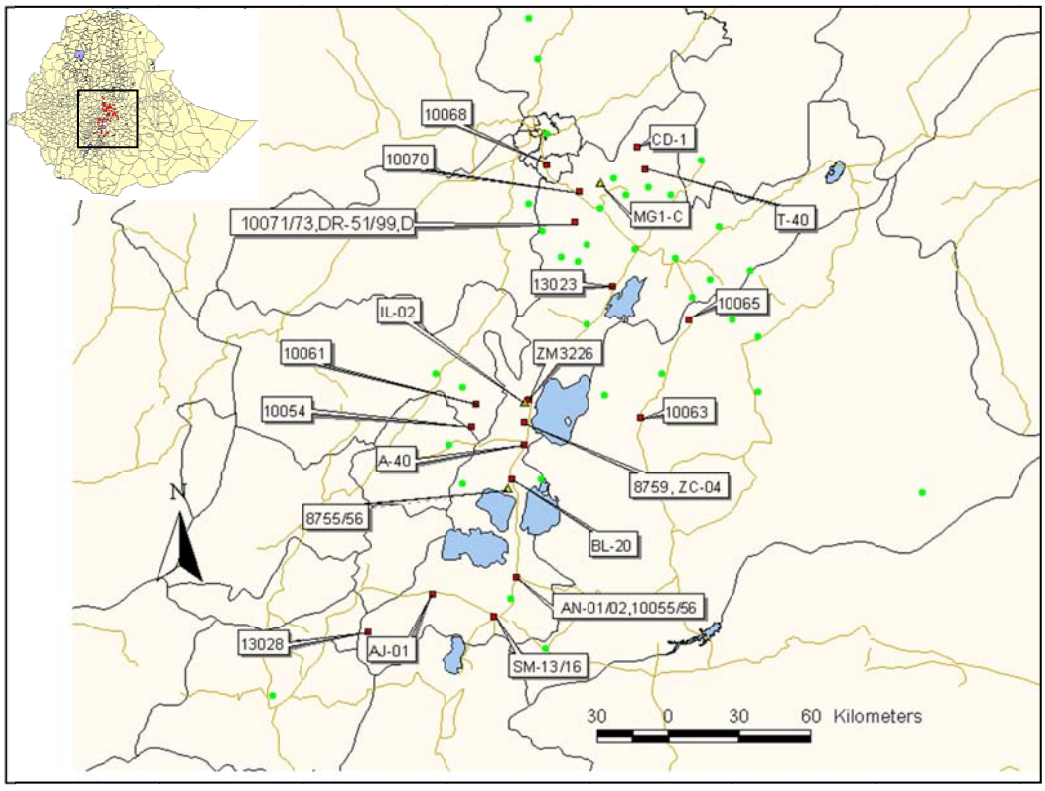

Figure 5.1 Map of Ethiopia showing the 20 locations where NDV isolates were obtained (in text boxes are sample ID, the triangles are villages and the squares are markets).

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5.4.2 Phylogenetic analysis of F and HN genes sequence

Complete sequencing of the F and HN genes revealed that the five isolates selected had gene lengths of 1659-bp and 1713 bp, respectively. Phylogenetic analysis of a sequence of the partial F gene (1047 bp), position 1-1047, and complete HN (1713 bp) of the five isolates, along with the two previous outbreak isolates ETH762 (chicken) and ETH303 (dove), is shown in Figures 5.2 and 5.3. Comparing complete nucleotide sequences of F protein gene, the five isolates from this study (indicated by black squares) are grouped in a cluster under genotype VI, branched with pigeon paramyxovirus-1 (PPMV-1) variants that belong to sub-genotype VIb, where also ETH303 branched (Fig 5.2). This was further confirmed on the HN gene sequence (Figure 5.3). The previous chicken isolate (ETH762) clustered with genotype VII (Figure 5.2).

The estimated evolutionary distance (maximal and minimal) for all the sequences/genotypes included in the present study is shown in Appendix B. The estimation of evolutionary divergence between selected sequences from genotype VI (including Ethiopian isolates in the present study and those from outbreaks) is shown in Table 5.3. Based on the sequence alignments and genetic distance comparisons, the five Ethiopian isolates analyzed clustered in a distinct group. Together they showed 94.6-98.8% nucleotide similarities i.e., the dissimilarity (maximum divergence) between Ethiopian genotypes was 5.4% (between ETHMG1C and ETH10073) suggesting an epidemiological linkage between places, even if they were from different areas, while the maximal distance between the present isolates and the reference PPMV1-clusters (from GeneBank), where also ETH303-dove is included in the tree, on complete F gene was more than 12%. The sequence divergence between the ETHMG1-C and ETH303 or JQ429293 (the two PPMV-1 in the cluster) varied from 12.2% to 13.8%, respectively (Table 5.3) suggesting a separate evolutionary path from PPMV-1 strains.

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Table 5.2 Origin of the sample, location and cleavage site motifs (112-117) of the NDV isolates included in this study (isolated during 2011).

No	Sample ID[a]	Woreda	Place	Area	Host	Motif of cleavage site
1	10071	Ada'a	Market	Dire	Chicken	G/RRHKR/FV
2	10073	Ada'a	Market	Dire	Chicken	G/RRHKR/FV
3	MG1-C	Ada'a	Village	Migra	Chicken	G/RRRKR/FV
4	8755	ATJK	Village	Maliyu	Chicken	G/RRQKR/FV
5	8757C	ATJK	Village	Maliyu	Chicken	G/RRRKR/FV
6	13028	Siraru	Market	Raphe	Chicken	G/RRQKR/FV
7	CD-1	Gimbichu	Market	Cafedonsa	Chicken	G/RRRKR/FV
8	BL-20	ATJK	Market	Bulbula	Chicken	G/RRRKR/FV
9	AJ-01	Shala	Market	Aje	Chicken	G/RRRKR/FV
10	10063	Asela	Market	Assela	Chicken	G/RRQKR/FV
11	10068	Akaki	Market	Akaki	Chicken	G/KRRKR/FV
12	10070	Akaki	Market	Akaki	Chicken	G/KRRKR/FV
13	10065	Dhera	Market	Dhera	Chicken	G/RRQKR/FV
14	10061	Mareko	Market	Qoshe	Chicken	G/KRRKR/FV
15	AN-01	Arsi Negele	Clinic	Arsinegele	Chicken	G/RRQKR/FV
16	AN-02	Arsi Negele	Clinic	Arsinegele	Chicken	G/RRQKR/FV
17	10055	Arsi Negele	Market	Arsinegele	Chicken	G/RRQKR/FV
18	10056	Arsi Negele	Market	Arsinegele	Chicken	G/RRRKR/FV
19	10054	Mareko	Market	Hudasa	Chicken	G/RRQKR/FV
20	13023	Lome	Market	Koka	Chicken	G/RRQKR/FV
21	11853-1[b]	Lome	village	Mojo	Pigeon	G/RRRKR/FI
22	ZM3226	ATJK	Market	Negalign	Chicken	G/RRRKR/FV
23	IL-02	ATJK	Village	Ilka village	Chicken	G/RRRKR/FV
24	SM-13	Shashemene	Market	Shasmene	Chicken	G/RRQKR/FA
25	SM-16	Shashemene	Clinic	Shasmene	Chicken	not clear
26	ZC-04	ATJK	Clinic	Ziway	Chicken	G/KRRKR/FV
27	8759	ATJK	Clinic	Ziway	Chicken	G/RRRKR/FV
28	3083	ATJK	Farm	Amba	Chicken	G/RRRKR/FV
29	8306	ATJK	Market		Chicken	G/RRRKR/FV
30	A-40	Ada'a	Market	AdamiT-1	Chicken	G/RRQKR/FV
31	D-60	Ada'a	Market	Dire-1	Chicken	G/RRQKR/FV
32	D-66	Ada'a	Market	Dire-1	Chicken	G/RRQKR/FV
33	DR-51	Ada'a	Market	Dire-2	Chicken	G/RRRKR/FV
34	DR-99	Ada'a	Market	Dire-2	Chicken	G/RRRKR/FV
35	T-40	Ada'a	Market	Tulidimitu-1	Chicken	G/RRRKR/FV

[a] samples originated from the same animal are not shown here

[b] outbreak cases in pigeon

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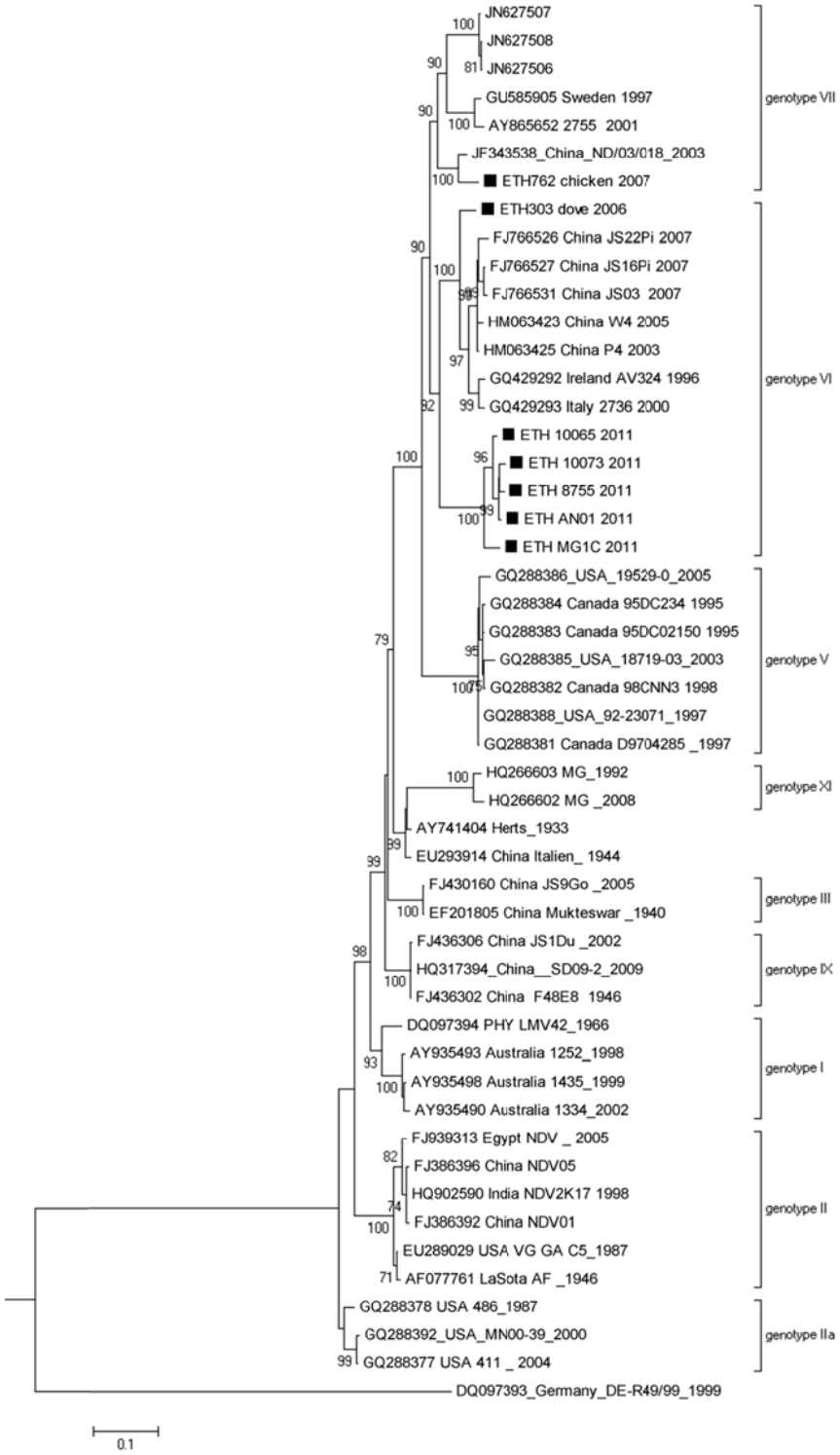

Figure 5.2 Phylogenetic tree of the nucleotide sequences of the Ethiopian NDV isolates based on 1047 bp of the F gene. Isolates described in this study are indicated by black squares. Genotypes are indicated at the right. Numbers at nodes indicate percentage value by bootstrap resampling of 1000 replicates.

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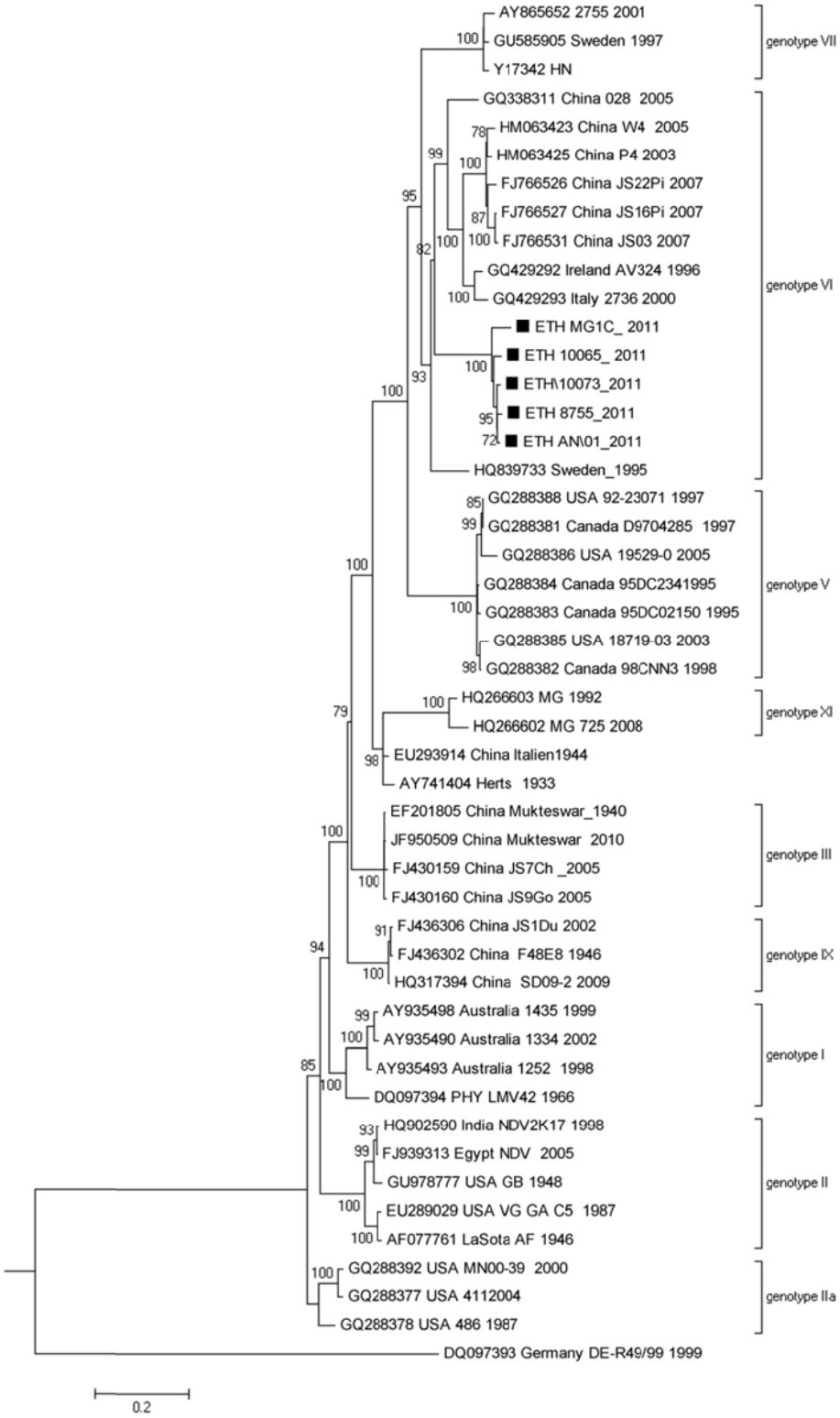

Figure 5.3 Phylogenetic tree of the nucleotide sequences of the Ethiopian NDV isolates based on 1713 bp of the HN gene. Isolates described in this study are indicated by black squares. Genotypes are indicated at the right. Numbers at nodes indicate percentage value by bootstrap resampling of 1000 replicates.

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Table 5.3 The number of base substitutions per site for sequence pairs of strains in genotype VI (included in the tree).

	ETH_10065	ETH_10073	ETH_8755	ETH_AN01	ETH_MG1C	ETH303	ETH762	FJ766526	FJ766531	GQ429292	GQ429293	HM063423	HM063425
ETH_10073	0.025												
ETH_8755	0.022	0.016											
ETH_AN01	0.018	0.013	0.012										
ETH_MG1C	0.042	0.054	0.053	0.047									
ETH303	0.118	0.128	0.120	0.124	0.122								
ETH762	0.148	0.161	0.151	0.149	0.154	0.125							
FJ766526	0.137	0.148	0.141	0.143	0.136	0.061	0.131						
FJ766531	0.138	0.145	0.138	0.142	0.135	0.060	0.134	0.027					
GQ429292	0.135	0.141	0.135	0.137	0.139	0.059	0.138	0.050	0.052				
GQ429293	0.134	0.144	0.137	0.138	0.138	0.055	0.130	0.052	0.052	0.017			
HM063423	0.133	0.143	0.135	0.137	0.135	0.057	0.134	0.025	0.023	0.046	0.046		
HM063425	0.128	0.138	0.130	0.132	0.130	0.050	0.125	0.018	0.016	0.038	0.038	0.011	
JF343538	0.133	0.145	0.139	0.136	0.134	0.111	0.041	0.115	0.117	0.118	0.112	0.115	0.106

The number of base substitutions per site between sequences are shown. Analyses were conducted using the Tamura-Nei model (Tamura *et al*., 1993). The analysis initially involved 50 nucleotide sequences (shown in Appendix B). Codon positions included were 1st+2nd+3rd+Noncoding. All positions containing gaps and missing data were eliminated. There were a total of 1047 positions in the final dataset.

5.5 Discussion

Newcastle disease is one of the most important and widespread enzootic disease in Ethiopia and remains a constant threat to village based chicken production and the emerging small scale poultry production. In this study Ethiopian isolates, obtained from suspected cases of chickens in villages, markets and clinics, were characterized at molecular level, by sequencing on F and HN genes and compared with other published sequences in GenBank. This represents the first molecular characterisation of NDV circulating between markets and villages in Ethiopia, and provides evidence of the existence of a sub-genotype, branched or sharing common ancestor with sub-genotype VIb of PPMV-1. The viruses were isolated from unvaccinated village chickens. The partial sequencing on the F gene (154bp) indicated that the isolates all belonged to virulent strains indicated by the cleavage site amino acid motif sequences ^{112}RRRKRF^{117}, ^{112}RRQKRF^{117}, ^{112}RRQKRF^{117}, ^{112}RRHKRF^{117} and

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^{112}KRRKRF^{117} (de Leeuw *et al*., 2005; OIE, 2008b). The presence of a number of basic residues in the fusion protein cleavage site has long been considered a key contributor to APMV-1 pathogenicity (Glickman *et al*., 1988; Seal *et al*., 1995). The epidemiological information obtained at villages during household surveys, where farmers reported high mortality of chickens with outbreaks of suspected ND, supports the conclusion that virulent NDV strains are endemic in Ethiopia. The first virus isolate, and those reported thereafter in Ethiopia, were attributed to velogenic virus, although their genetic characteristics were not documented (Lefevre and Martel 1975; Bawke *et al*., 1991; Nasser, 1998). Since then the disease has become endemic with outbreaks occurring all over the country. But whether these outbreaks have been caused by genetically similar viruses or from multiple introductions over periods of time were not possible to ascertain hitherto. Previous outbreak investigation report of genotype VII in the western part of Ethiopia (ETH762) and the finding in the present study of viruses that clustered in genotype VI, suggests multiple infections with different virus genotypes that were introduced at different times. Moreover, the direct sequences amino acid motif of the F gene on those RT-PCR positive swab samples from apparently healthy chickens at markets and villages (Chapter 3) demonstrated the existence of low virulent viruses with variable amino acid sequences at the cleavage sites confirming the possible existence of different pathotypes.

The complete F gene sequencing and phylogenetic analysis on selected isolates from geographically distinct markets and villages in the study area showed that circulating virus strains in the area studied grouped in a single genetic cluster (with maximum nucleotide dissimilarity of about 5.4%), as also confirmed on HN gene, sharing a common ancestor with a NDV variant that commonly affects pigeons, PPMV-1, in sub-genotype VIb (Figure 5.2). The bootstrap value supporting the phylogenetic relationship of these two clusters is relatively high (92%). The PPMV-1 strain is genetically distinguishable from the APMV-1 (Aldous *et al*., 2003) with specific monoclonal antibody patterns that do not agglutinate chicken RBC (Ujavari *et al*., 2003) but may result in clinical disease in chickens (Kommers *et al*., 2001). The genetic distance between the reference PPMV-1 cluster and the current circulating strains in our study area indicates, although the viruses have common ancestor, the Ethiopian isolate clustered well apart to be considered possibly in a separate sub-genotype. In fact, within genotype VI the distances between sub-genotypes VIc and VIf (0.061) or between VIf and VIb (0.054) are larger than the distances between genotype III and IV (0.041) suggesting that genotype VI may need further separation (Miller *et al*.,

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2010b). Hence, although they appeared closely clustering the Ethiopian isolates in the present study could also be subjected for reconsideration, in light of the new ways of genotype classification suggested recently (Diel *et al*., 2012).

The close genetic similarities (with nucleotide divergence < 5%) of the isolates from different locations in the present study, could provide some evidence of epidemiological linkage between locations, showing that the isolated strains may be circulating widely in the study area, and perhaps further a field in Ethiopia. Markets might have played a major role in the spreading of the virus, as most isolates were obtained at market places that are attended by farmers and traders are involved in moving live chickens between markets (Chapter 7). Nevertheless, the Ethiopian NDVs previously isolated from an outbreak in western part of Ethiopia, near Sudan (ETH762), clustered within genotype VII, distinctly apart from those in the present study and more closely related with strains from China (JF343538_China_ND/03/018_2003) (Fig 5.2), which had 95% nucleotide identity with the Sudanese strains from recent outbreaks (GQ258669, GQ258672) (Hassan *et al*., 2010). The laboratory result from VLA corroborated this, showing that ETH762 had 99.2% identity with ETH764 (not shown on the tree), which in turn had 97.6% similarity with an isolate from Sudan in 2005. Additionally, from molecular investigation on swab samples from apparently healthy chickens at villages and markets, there was an indication of circulation of both genotype VII and VI viruses, based on partial F gene (239 bp) sequencing, but this was not demonstrated with certainty as the sequences analysed were very short. Whether the strains isolated in the present study are representative of those elsewhere in Ethiopia requires further investigation covering a wider area.

To date the genotypes or lineages investigated and characterized from Africa have included genotypes VIIb, VIId and VIII from Southern Africa (Herczeg *et al*., 1999; Abolnik *et al*., 2004; Fringe *et al*., 2012); a novel genotype close to VIa (up to 89% similarity) from Uganda (Otim *et al*., 2004); genotypes V and VIa from Tanzania (Yongolo *et al*., 2011); lineage 5a (VII) from Sudan (Hassan *et al*., 2010); virulent genotype II from Egypt (Mohammed *et al*., 2011); and lineage 4b and a suggested novel lineage 7 (including those previously described as lineage 5 in genotype VII) from pigeon and rural domestic chickens in western Africa (Snoeck *et al*., 2009; Cattoli *et al*., 2010; Van Borm *et al*., 2012). This shows that virus in those respective regions tended to fall into similar or closely related genotypes, signifying temporal or spatial association of the circulating strains in the spread of ND. Because of

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geographic proximity, one would expect circulation in Ethiopia of similar strains of virus to those in the eastern African region, as was the case in western or southern Africa. However, Ethiopian isolates in the present study appeared to be different, showing no clear common ancestor with those reported in East Africa. This may suggest that the viruses responsible for disease in our study area might have been introduced from outside Africa rather than from neighbouring countries such as Kenya or Sudan. It could be possible that an independent evolution has taken place following a much earlier introduction of a strain that was common in the region some time back. The closer proximity of divergence of the tree for the contemporary Ethiopian isolate and the PPMV-1, predominantly from Asia and Europe, supports this possibility (Fig 5.2 & 5.3). Nevertheless, the Kenyan NDV strain chicken/Kenya/KRC-139/94 (AY505066.1), sequenced on the polymerase (L) gene (6615 bp) exhibited 95% similarity with PPMV-1 (GQ429292), upon BLAST search (Zhang *et al*., 2000), with which also the Ethiopian isolates in the present study branch. The report from Sudan of ND in chickens in 1970 also confirmed the existence of a more PPMV-1 related virus (AY151383-84) and phylogenetic analysis suggested that PPMV-1 (VIb/1) could be of African origin (Ujvari *et al*., 2003). Whether there is any epidemiological link between Ethiopian and old Sudanese strains is difficult to ascertain. Recently characterized (F gene) the Sudanese NDV from 2003-2006 outbreaks were rather attributed to lineage 5d (VII) (Hassan *et al*., 2010), more related to the strain (ETH762) isolated from an outbreak near Sudanese border, suggesting a possible epidemiological relationship. Studies based on the complete genome sequences of strains isolated from different geographic regions of Ethiopia are necessary to understand their genetic relatedness with NDV strains circulating in other parts of Africa and the rest of the world.

During our field study we also investigated a disease outbreak in wild doves (*Streptopelia* spp.), which was later determined to be caused by PPMV-1 with cleavage site motif "G/RRRKR/FI" and found to be closely related to ETH303. Based on this analysis, the close phylogenetic proximity of the isolates in the present study to PPMV-1 variants would suggest that viral transmission may occur between wild birds and domestic poultry in the study area. Recently a few chicken samples from Ethiopia, sent to the VLA for NDV diagnosis, were attributed to PPMV-1 (VLA, diagnostic results, 2009 & 2010), although the magnitude of this problem was not established. PPMV-1 can be transmitted from infected pigeons to in-contact chickens, with cloacal virus excretion up to 31 days post-infection (dpi) and antibody response to the virus demonstrating virus replication in chickens (Alexander and Parsons,

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1984). Several outbreaks in domestic commercial chickens of Great Britain were attributed to the contamination of feed by faeces of PPMV-1 infected pigeons (Alexander *et al*., 1984). Wambura (2010), based on antibody assessment, has documented that pigeons are susceptible to APMV as well. It was shown that pigeons infected with highly pathogenic APMV-1 strains under experimental infections, seroconverted and shed virus up to 24 dpi without evident clinical signs (de Oliveira Torres Carrasco *et al*., 2007). Phylogenetic analyses have identified velogenic NDV isolates from pigeons and also implicated migrating cormorants as the likely source of some ND outbreaks in poultry (Banerjee *et al*., 1994; Ujvari *et al*., 2003; Aldous *et al*., 2004). In addition, PPMV-1 strains were responsible for epizootics in chickens in the Middle East and probably in Asia in the 1960/70s (Ballagi-Pordany *et al*., 1996) and a third panzootic during the 1980s (Kelata *et al*., 1985). Considering the potential risk of village chickens being contaminated by wild bird carriers of PPMV-1 and APMV-1, close monitoring and surveillance of NDV wild birds is required, since these species may serve as a bridge between village chickens and commercial chicken operations.

In the present study virulence was judged based on the molecular characterization of the F gene cleavage sites. Sequence analysis of the F protein cleavage site can be used to predict potential pathogenicity of NDV in complementary with conventional methods such as mean death time (MDT) and intracerebral pathogenicity index tests (ICPI) (Seal *et al*., 1995; Marin *et al*., 1996; Panda *et al*., 2004). West African virus strains with virulent amino acid motifs had ICPI ≥ 1.7, showing that these two pathogenicity assessment parameters coincided (Cattoli *et al*., 2010). Nevertheless, as F protein cleavage site alone cannot confer virulence (Yongol *et al*., 2011), it is advisable to consider also other *in vivo* tests such as ICPI and MDT on these isolates from present study.

In conclusion, the present study provided valuable information on virus strains circulating in village chickens in our study area in Ethiopia, which is different from what has been recorded previously in Ethiopia from two outbreaks investigated. Despite the limited number of isolates (five) characterized and the limited area covered, the study revealed circulation of similar virus strains between markets and villages. The study lays the foundation for broader molecular epidemiological studies to describe the evolution of the disease in the region and further field in Africa. It has been postulated that new lineages or sub-lineages are emerging as the consequence of continuous evolution of NDV (Aldous *et al*., 2003) or by introduction from other areas, possibly via trade movements. Further investigation and continuous

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surveillance would provide additional knowledge, bridge the existing epidemiological information gaps in Ethiopia, and generate information relevant to the region and Africa.

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CHAPTER SIX

EVALUATION OF ENZYME-LINKED IMMUNOSORBENT ASSAYS AND HAEMAGGLUTINATION INHIBITION TESTS FOR THE DIAGNOSIS OF NEWCASTLE DISEASE VIRUS INFECTION IN VILLAGE CHICKENS USING A BAYESIAN APPROACH

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6.1 Abstract

Newcastle disease in village chicken is endemic in Ethiopia with significant economic importance. The sensitivity (*Se*) and specificity (*Sp*) of the blocking enzyme-linked immunosorbent assay (bELISA, Svanova Biotech), indirect ELISA (iELISA, Laboratoire Service International) and haemagglutination inhibition (HI) test for Newcastle disease virus (NDV) antibody detection were evaluated in a Bayesian framework in the absence of a gold standard test, on sera collected from unvaccinated chickens kept under the village production system in household flocks and at markets in Eastern Shewa zone, Ethiopia. The bELISA had both the highest *Se* (96.3%; 95% posterior credible interval (PCI): 88.1; 99.8%), and the highest *Sp* (98.9%; 95% PCI: 97.8; 99.9%), while the HI had a *Se* of 81.6% (95% PCI: 71.8, 91.9%), and a *Sp* of 96.1% (95% PCI: 95.1; 96.6%). The iELISA also had high *Se* (95.2%; 95% CPI: 88.5; 99.0%) but had very poor *Sp* (8.9%; 95% PCI: 6.4, 11.8%). The conditional correlation between the tests for both sensitivity and specificity clustered around zero, indicating conditional independence. The use of bELISA in screening and surveillance for NDV antibodies is indicated given its high *Se* and *Sp*, in addition to its ease of automation to handle large numbers of samples compared to HI. The latter can be used as confirmatory test where an ELISA test with moderate or low specificity is used.

Key words: Newcastle disease, chickens, diagnostic test evaluation, Bayesian framework, enzyme-linked immunosorbent assay, haemagglutination inhibition test

6.2 Introduction

Newcastle disease (ND), caused by avian paramyxovirus virus type 1 (APMV-1), is a poultry disease with significant economic importance in the world in both commercial settings and village scavenging production systems such as the one widely practised in Ethiopia (Alexander *et al*., 2004; Cattoli *et al*., 2011). The control of the disease in village chicken flocks is hampered by low veterinary capacity and diagnostic coverage, lack of clear control policies, lack of vaccine suitable for tropical rural conditions (in terms of dosage and thermal stability) and likely also due to low prioritization of development of the sub-sector.

Diagnosis of ND is done by isolation of the virus in embryonated chicken eggs in addition to using serological tests such as various enzyme-linked immunosorbent assay (ELISA) and

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haemagglutination inhibition (HI) tests (OIE, 2008b). During recent decades several polymerase chain reaction (PCR) based assays have been developed for the detection of paramyxovirus nucleic acids in a variety of clinical samples (Gohm *et al*., 2000; Cattoli and Monne, 2009). While isolation of the virus in embryonated eggs is regarded as a 'gold standard' it takes a relatively long time to culture virus or supply of SPF eggs may also be a problem. The use of molecular diagnostic assays is not within reach of many laboratories in developing countries because of the equipment, reagents and expertise required. Therefore, serological tests such as ELISA and HI are often relied upon for the diagnosis and surveillance of ND, indirectly through the detection of antibodies produced following infection by ND virus (NDV). However, these tests have limitations due to uncertainty regarding their sensitivity and specificity, as well as, lack of reproducibility under different laboratory conditions (Beard and Wilkes, 1985; Schelling *et al*., 1999; de Wit *et al*., 2007). For instance, NDV has been reported to show some degree of cross-reactivity in HI tests with several of the other avian paramyxovirus serotypes, especially APMV-3 psittacine isolates, using polyclonal antisera (Alexander *et al.,* 1983; Lipkind and Shihmanter, 1986; Adair *et al*., 1989). It was reported that ELISA proved more sensitive and rapid but less economic than HI when used for detection of antibodies against NDV, mostly in vaccinated birds (Adair *et al*., 1989; Cadman *et al.,* 1997; Tabidi *et al*., 2004). The performance of ELISA and HI tests under conditions of field exposure, such as in village chickens, has so far not been well investigated.

The sensitivity (*Se*) and specificity (*Sp*) of a test are usually estimated by comparison with a reference (gold standard) test that is assumed to reveal unambiguously the true state of animals with respect to disease (Greiner and Gardner, 2000). However, the true state is rarely known because the results of the available diagnostic tests do usually not reflect the true state without uncertainty. The test performance parameters (*Se* and *Sp*) that define probabilistic relationships between test results and true epidemiological states can be estimated in a Bayesian framework. Once these parameters have been estimated, valid inferences on prevalences can be made (Enue *et al*., 2000). The Bayesian framework is particularly suited for test performance evaluation because prior scientific information about the *Se* and *Sp* of the tests and prior information about the prevalence of the sampled populations can be incorporated (Branscum *et al*., 2005). The prior distributions could be based on published values from previous studies or from expert opinion (Suess *et al*., 2002). Basically, distinct tests are assumed to be conditionally independent and the theoretical sensitivities and

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specificities of tests in combination are calculated directly from individual test values. However, for tests that measure similar biologic processes such as serum antibody responses to infectious agents, it is logical to expect that test results will be not be independent, conditional on an animal's true status (Gardner *et al*., 2000). It is importance to address such dependence between the sensitivities or specificities of pairs of tests because it affects the *Se* and *Sp* of tests when used in combination (Greiner and Gardner, 2000).

The objective of this study was to evaluate two ELISAs and HI tests for the detection of NDV antibodies in chickens under the village poultry production system in Ethiopia using a Bayesian framework.

6.3 Materials and methods

6.3.1 Serum samples

Chicken sera used in this study were collected during structured, random, multistage household and markets surveys of village chickens in Eastern Shewa zone, Ethiopia between September 2009 and September 2010. The sera were collected from household chickens of indigenous breeds, in villages and at selected markets in the study area. The chickens were kept in a scavenging village production system. None of the study households vaccinated their chickens against ND (Chapter 3). Only grower and adult chickens older than 2 months of age were included in the study. In villages a total of 1899 sera which constituted the "household dataset" were collected during two seasons of the year (n = 1018 for wet season and n = 881 for dry season) (detail is described in Chapter 3) and tested with a blocking ELISA (bELISA) and HI tests. Sera from markets, constituting the "market dataset" were obtained during two different seasons (n = 204 for wet season and n = 233 for dry season) (detail is described in Chapter 2) and tested with the bELISA, an indirect ELISA (iELISA) and HI tests. The samples from each season were considered to be from separate populations (wet season = population one; dry season = population two) in this study. The analysis of the serum was performed at the National Animal Health Diagnostic and Investigation Center (NAHDIC), Ethiopia.

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6.3.2 Blocking ELISA

Blocking ELISA was performed using Svanovir™ NDV-Ab test kits (Svanova Biotech, Uppsala, Sweden) using the procedure described by manufacturer and validated by Czifra *et al*. (1996). The test was developed to detect specific serum antibodies against a well-conserved epitope present in APMV-1 strains. Briefly, NDV-coated ELISA plates were first incubated with the samples, allowing NDV specific antibodies present in the sample to bind to epitopes. If NDV specific antibodies were absent, the epitopes remained free. After washing, horseradish peroxidase (HRP) conjugate monoclonal antibody (mAb) was added to the wells and the binding of the conjugate mAb was visualized with a substrate solution. A negative result was indicated by a strong colour change. The sample and control optical density (OD) values were read using an ELISA reader at 450 nm.

The antibody titres were calculated as percentage inhibition (PI) for the positive control as well as the samples, using the formula

$$PI = [(OD_{Negative\ control} - OD_{Sample}) / OD_{Positive\ control}] \times 100/OD_{Negative\ Control}$$

The test was considered valid when $OD_{Negative\ control}$ was greater than 0.60 and a PI of greater than 40. All sera that blocked the binding of the specific monoclonal antibody with a 40% inhibition (PI >40) were considered bELISA positive.

6.3.3 Indirect ELISA

The LSIVET AIV NDV® ELISA was obtained from Laboratoire Service International (LSI, Lissieu, France) and is based on the principle of indirect ELISA (iELISA) and designed to be used on individual poultry serum. The iELISA works on the principle of recognition of anti-NDV antibodies, attached to a plate coated with viral antigen (nucleoprotein 1), by antibodies produced in another species against chicken antibodies. Briefly, samples were added to the NDV antigen coated plate. If present, specific antibodies against NDV bound to antigen forming antigen-antibody complexes. After washing anti-chicken IgY/HRP-labelled conjugate was added, binding to any chicken antibodies attached to the plate. Unbound conjugate was removed by washing, after which a chromogen substrate was added. This anti-chicken antibody is conjugated to an enzyme (peroxydase) that catalyses a reaction, causing a change of colour which was read quantitatively on a photospectrometer at 405nm.

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For each tests the sample-to-positive (S/P) ratios were calculated from absorbance values by the formula:

$$S/P \text{ ratio} = [OD_{Sample} - OD_{Negative\ control\ mean}] / [OD_{Positive\ control\ mean} - OD_{Negative\ control\ mean}]$$

The test was considered valid if OD of positive control >0.5 and (OD mean Positive control/OD mean Negative control) > 6. All samples with S/P ratio greater than 0.234 were considered positive (manufacturer's recommendation).

6.3.4 Haemagglutination inhibition test

The HI test is based on the principle that the haemagglutinin on the viral envelope can bring about the agglutination of chicken red blood cells (RBC) and that this can be inhibited by specific antibodies (Alexander *et al*., 2004). The test was performed as described by the World organization for Animal Health (OIE, 2008b). The antigen used was a commercial NDV LaSota strain provided by Laboratoire Service International (LSI), France and reconstituted in sterile distilled water, while positive and negative control NDV strain La Sota antisera were procured from GD Animal Health Service Ltd, Deventer, The Netherlands. Antigen was diluted to contain 4 haemagglutination units (HAU). The test was performed on V-bottom microtiter plates. The presence or absence of agglutination was accurately assessed by tilting the plates. Only those wells in which the RBCs streamed at the same rate as the control wells (containing RBCs and PBS only) were considered to show inhibition. The titres were expressed as $\log_2$ of the reciprocal of the highest dilution of serum giving complete inhibition of 4 HAU. Sera with HI antibody ≥4 $\log_2$ were considered positive for NDV antibody (OIE, 2008b).

6.4 Prior information, assumptions and test evaluation

Prior information for the test parameters (*Se* and *Sp*) were obtained from published papers (Czifra *et al.,* 1996; Koch *et al*., 1998; Gohm *et al.,* 1999; Hauslaigner *et al*., 2009) for the bELISA and HI test, and manufacturers' claim (Laura Fournier, 2011, *personal communication* for iELISA) while information on the prevalence was obtained from available literature (Zeleke *et al*., 2005b; Regassa *et al*., 2007; Getachew, 2009) and beta prior distributions were elicited using beta buster software (www.epi.ucdavis.edu/diagnostictest/). Prior distribution of the *Se* and *Sp* of the three tests and the population prevalence were

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modelled as beta (α, β) distributions where parameters α and β were derived from the most likely value (mode) and the 5th or 95th percentile of the *Se*, *Sp* and prevalence (Table 6.1). Because the ELISAs and HI all detected NDV antibodies, we regarded the three tests as possibly dependent. We assumed constant test performances irrespective of season or survey type so that test accuracy estimates were interpreted as estimates of the average sensitivity or specificity across populations (Branscum *et al.,* 2005).

The *Se* and *Sp* of the ELISAs and HI test and the seroprevalences were estimated in a Bayesian framework using the model described in Branscum *et al.,* (2005) modified to fit the specific sampling design of our study. In this modified version, the *Se* and *Sp* parameters for bELISA and HI were estimated using both data from household and market while that of iELISA was based on market data only, although all the tests were treated in a single model. Since the tests measured broadly the same biological process (antibodies to NDV), models with conditional dependencies within pairs of tests were considered. Moreover, models considering two distinct populations (wet season and dry season, irrespective of the survey type) and models considering four distinct populations (one distinct population for each season and survey type) were fitted to the data. The model script is shown in Appendix C. The models were fitted using the freeware programme WinBUGS version 1.4 (Spiegelhalter *et al*., 2003). The program uses Markov Chain Monte Carlo (MCMC) sampling to obtain a Monte Carlo (MC) sample from the posterior distribution. One hundred thousand iterations were used to make the posterior inference, with the initial 2000 iterations discarded as burn-in. Convergence of the MCMC chains was assessed by visual inspection of Gelman-Rubin diagnostic plots using two MCMC chains with different initial values (Toft *et al*., 2007). Additionally, density and trace plots were checked to assess the convergence of the two chains. The deviance information criterion (DIC) was used to compare models, with a smaller value indicating better fit.

The posterior mean and 95% posterior credible intervals (PCI, 2.5 and 97.5 percentiles), for each of the parameter estimates (*Se*, *Sp* and true prevalence) were presented.

The conditional correlation parameters (*rho*) of the tests on the status of the animals were also estimated for the models; *rho*D and *rho*Dc represent the conditional correlation between the test outcomes among diseased animals (for sensitivity) and healthy animals (for specificity), respectively. Ideally the presence of pathogen is used to decide the disease status

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of an animal. However, since we evaluated serological tests, for the definition of disease status, the presence or absence of antibodies to NDV was considered as the indicator for the status of an animal. The tests were considered conditionally independent on the status of an animal if the rho values were equal to or clustered around zero (Branscum *et al.,* 2005).

6.5 Sensitivity analysis

Sensitivity analysis, which involved a non-informative prior (uniform distribution) as well as the given prior, were performed alternatively for the sensitivity and specificity of each test as well for expected prevalence to assess the influence of prior distributions on parameter estimates. Overlapping of PCI was considered as evidence of model robustness.

Table 6.1 Available information on test sensitivity (*Se*) and specificity (*Sp*) and prevalence (*pi*), and corresponding prior distributions.

Parameter	Prior knowledge			Prior distribution			
	Mode (%)	5th centile	95th centile	*alpha*	*beta*	Mean (%)	95% PI
Se bELISA	98.0	85.0		23.35	1.46	94.1	82.3; 99.6
Se iELISA	95.0	85.0		36.7	2.87	92.7	88.0; 98.5
Se HI	85.0	70.0		23.9	5.04	82.6	67.1; 93.8
Sp bELISA	99.0	90.0		34.17	1.36	96.2	88.0; 99.8
Sp iELISA	99.0	80.0		14.52	1.14	92.7	76.1; 99.7
Sp HI	99.0	90.0		34.17	1.36	96.2	88.0; 99.8
*pi*1	10.0		50.0	1.49	5.38	21.6	1.9; 56.4
*pi*2	15.0		50.0	1.93	6.25	23.6	3.2; 55.8

6.6 Results

6.6.1 Descriptive results

Cross classification of the serological test results is shown in Table 6.2. The bELISA and HI had a Kappa, measure of test agreement, value of 0.704 (95% CI: 0.645 - 0.764) for the household data set (n = 1899) that was considered as good or substantial agreement according to (Landis and Koch, 1997). For the market samples the values were less than 0.01 for

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iELISA compared with the others (bELISA and HI) to be considered as poor or slight agreement.

Table 6.2 Cross classified serological test results from chicken serum samples from households and markets in Eastern Shewa zone, Ethiopia during September 2009 and May 2010.

Data set	n*	Serological tests		Combination of test results							
				Population one (Wet season)				Population two (Dry season)			
		Test1	Test2	R++	R+-	R-+	R--	R++	R+-	R-+	R--
1 [a]	1899	bELISA	HI	41	15	37	925	73	23	10	775
2[b]	437	iELISA	bELISA	13	0	179	12	14	0	207	12
2 [b]	437	iELISA	HI	19	173	2	10	33	188	2	10
2 [b]	437	bELISA	HI	7	6	14	177	7	7	28	191

*number of serum samples tested

[a]household dataset

[b]market data set

6.6.2 Evaluation of test performance

Several alternative models for estimating test performances parameters and prevalences were fitted to the available data (Table 6.3). According to the DIC, and favouring parsimony when models had similar DICs, Model 4 (Table 6.3), which considered four distinct populations and no conditional dependence among tests, was selected. This model converged very well according to all convergence criteria (chain mixing, autocorrelation, unimodal posterior distribution). In this model, the *Se* of bELISA, HI and iELISA was estimated at 96.3% (95% PCI: 88.1; 99.8%), 81.9% (95% PCI: 71.8, 91.9%) and 95.2% (95% PCI: 88.5; 99.0%), respectively (Table 3). Specificity estimates were high for bELISA and HI: 98.9% (95% PCI: 97.8; 99.8%) and 96.1% (95% PCI: 95.1; 96.9%), respectively, but considerably low for iELISA: 8.9 (95% PCI: 6.4; 11.8%). Posterior estimates for NDV seroprevalence in households were 5.0% (95% PCI: 3.5; 6.7%) during the wet season and 10.2 (95% PCI: 8.0; 12.6%) during the dry season, while for the markets, posterior prevalence estimates did not differ between the two seasons: 5.8 % (95% PCI: 2.6; 10.0%) during the wet season and 5.8 (95% PCI: 2.6; 10.2%) during the dry season. In general the parameter posterior estimates were quite close to prior distribution mean except for prevalences and iELISA *Sp*. The

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important discrepancy between the prior and posterior distributions for iELISA *Sp* suggests that posterior distributions were not overly influenced by prior distributions.

Table 6.3 The posterior mean and 95% posterior credibility interval (PCI), under different model specifications, for the sensitivity and specificity of the bELISA, iELISA and HI tests, the population prevalence and the conditional covariance on animal true status, assuming four separate populations.

Parameter	Model 1[a]		Model 2[b]		Model 3[c]		Model 4[d]	
	mean (%)	95% PCI (%)	mean (%)	95% PCI (%)	mean (%)	95% PCI (%)	mean (%)	95% PCI (%)
Se bELISA	95.5	86.1; 99.7	96.0	87.1; 99.7	96.0	85.9; 99.8	96.3	88.1; 99.8
Sp bELISA	98.5	96.5; 99.8	99.1	97.6; 99.9	98.9	97.8; 99.8	98.9	97.8; 99.8
Se Hi	78.7	67.5; 90.4	80.3	70.4; 91.2	81.7	71.4; 92.1	81.9	71.8; 91.9
Sp HI	95.5	93.2; 96.6	96.1	95.2; 97.0	96.1	95.2; 97.1	96.1	95.1; 96.9
Se iELISA	95.1	88.3; 99.0	95.2	87.6; 99.0	95.6	89.1; 99.1	95.2	88.5; 99.0
Sp iELISA	8.9	6.3; 11.8	8.9	6.4; 11.8	8.8	6.3; 11.8	8.9	6.4;11.8
*pi*1	5.5	1.9; 9.9	6.2	2.8; 10.6	5.8	2.6; 10.3	5.8	2.6; 10.0
*pi*2	5.4	1.9; 9.9	6.1	2.8; 10.8	5.9	2.6; 10.9	5.8	2.6;10.2
*pi*3	4.5	2.3; 6.6	5.1	3.6; 6.9	5.0	3.5; 6.8	5.0	3.5; 6.7
*pi*4	10.0	7.2; 12.7	10.4	8.1; 12.8	10.2	8.0; 12.6	10.2	8.0;12.6
*rho*D	1.71	-0.72; 7.67	1.30	-0.06; 4.45	0.97	-1.14; 4.61		
*rho*Dc	0.48	-0.02; 1.81	-0.07	-0.44; 0.13	-0.18	-0.15; 0.18		

[a] modified model combining all the tests and bELISA/HI dependent (DIC=150.41)

[b] modified model combining all the tests and iELISA/HI dependent (DIC=151.52)

[c] modified model combining all the tests and bELISA/iELISA dependent (DIC=152.44)

[d] modified model combining all the tests with no dependence assumed (DIC=150.95)

*pi*1 = Prevalence wet season (markets) *pi*2 = Prevalence dry season (markets)

*pi*3 = Prevalence wet season (households) *pi*4 = Prevalence dry season (households)

Table 6.4 shows the results from analysis where the two data sets were combined (along seasons) in the 2 population model with only two prevalence estimates. The parameter estimates were fairly similar to the four population approach with slightly higher DIC.

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Table 6.4 The posterior mean and 95% posterior credibility interval (PCI) of the sensitivity and specificity the bELISA, iELISA and HI tests, the conditional covariance on animal true status and the population prevalence from two population model fitted to the full data sets.

Parameter	Model1[a]		Model2[b]		Model3[c]		Model4[d]	
	mean (%)	95% PCI (%)	mean (%)	95% PCI (%)	mean (%)	95% PCI (%)	mean (%)	95% PCI (%)
Se bELISA	94.7	84.3; 99.6	93.7	81.8; 99.5	94.3	83.4; 99.5	94.4	83.9; 99.6
Sp bELISA	98.4	96.0; 99.8	99.0	97.9; 99.9	99.1	98.0; 99.9	99.0	97.9; 99.9
Se Hi	78.2	67.1; 90.0	81.0	71.1; 91.5	79.6	70.1; 90.6	81.4	71.4; 91.7
Sp HI	95.5	93.5; 96.8	96.3	95.2; 97.4	96.2	95.2; 97.3	96.2	95.2; 97.3
Se iELISA	95.2	88.5; 99.0	95.8	89.7; 99.2	95.5	89.3; 99.0	95.3	88.8; 99.0
Sp iELISA	8.9	6.4; 11.8	8.9	6.4; 11.8	9.0	6.4; 11.9	9.0	6.4; 11.9
*pi*1	4.6	1.9; 6.7	5.3	3.7; 7.1	5.4	3.8; 7.2	5.2	3.7; 7.0
*pi*2	8.9	6.0; 11.6	9.6	7.4; 12.0	9.7	7.5; 12.0	9.5	7.4; 11.8
*rho*D	1.28	-1.4; 6.5	0.82	-1.1; 4.1	1.38	-0.1; 4.5		
*rho*Dc	0.61	0.01; 2.2	-0.20	-0.7; 0.2	-0.09	-0.5; 0.1		

[a] two population model with full dataset and all the tests (bELISA/HI dependent) (DIC=153.07)

[b] two population model with full dataset and all the tests (iELISA/HI dependent) (DIC=154.63)

[c] two population model with full dataset and all the tests (bELISA/iELISA dependent) (DIC=153.59)

[d] two population model with full dataset and all the tests (no dependence assumed) (DIC=153.14)

*pi*1 = Prevalence wet season *pi*2 = Prevalence dry season

6.6.3 Test dependence

The posterior estimates of conditional covariance, rhoD and rhoDc, were very low (Tables 6.3 and 6.4). In all models their 95% PCI included 0, so one could not reject the null hypothesis that the test results were independent of each other conditional on the true serological status of an animal. Selection of the conditional independence model was therefore justifiable.

6.6.4 Sensitivity analysis

Sensitivity analysis was performed for the selected models (Table 6.3, model 4) and the estimates obtained for posterior distributions with and without informative priors (Table 6.5) remained fairly similar to the informed models. The use of non-informative priors for *Se* and

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Sp did not affect posterior estimates of the parameters, as evidenced by overlapping PCIs, except that a highly informative prior for *Se* of bELISA was required for the model to converge well (Table 6.5, model 4). According to the sensitivity analysis, the model is robust to provide inference on the test parameters for bELISA, IELISA, HI test, and prevalence parameters. When a non-informative prior was used for *Se* of bELISA, the posterior parameter estimates went down while the DIC increased. But with highly informative priors, the parameter estimates improved, with improved convergence, while the DIC relatively went up compared to other models and the prevalence estimate based on market data set slightly increased (Table 6.5, model 4).

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Table 6.5 Sensitivity analysis: posterior estimates for the Se and Sp of bELISA, HI and iELISA, and prevalence, in a four population Bayesian conditional independence model using non-informative priors for each parameter.

Parameter	Model 1 DIC=150.85		Model 2 DIC=150.96		Model 3 DIC=150.91		Model 4 DIC=153.12		Model 5 DIC=151.00		Model 6 DIC=151.31		Model 7 DIC=150.90		Model 8 DIC=150.88		Model 9 DIC=145.74	
	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI	Mean (%)	95% PCI
Se bELISA	96.6	88.8; 99.8	96.4	88.1; 99.8	96.6	89.1; 99.8	90.3	70.4; 98.6	96.3	87.9; 99.8	96.2	87.8; 99.8	96.5	88.6; 99.8	96.3	87.8; 99.8	96.4	88.4; 99.8
Sp bELISA	98.8	97.8; 99.8	98.9	97.8; 99.8	98.8	97.8; 99.8	98.9	97.9; 99.9	98.9	97.8; 99.8	99.0	97.7; 99.9	98.9	97.8; 99.8	98.9	97.8; 99.8	98.9	97.8; 99.8
Se HI	82.4	72.1; 92.4	82.0	71.8; 91.9	82.6	72.2; 92.6	81.3	71.4; 91.6	81.7	71.4; 92.1	80.9	68.7; 96.4	81.9	71.8; 92.0	81.9	71.7; 92.0	82.3	71.8; 92.3
Sp HI	96.1	95.1; 96.9	96.1	95.2; 97.1	96.1	95.1; 96.9	96.5	95.3; 98.4	96.1	95.2; 97.0	96.1	95.2; 97.0	96.0	95.1; 96.9	96.1	95.2; 97.0	96.1	95.2; 96.9
Se iELISA	95.2	88.4; 99.0	95.2	88.5; 99.0	95.1	88.4; 99.0	98.9	88.6; 99.0	95.2	87.5; 99.0	95.2	88.6; 99.0	95.2	88.5; 99.0	95.5	83.6; 99.9	95.1	88.3; 99.0
Sp iELISA	8.9	6.4; 11.8	8.9	6.4; 11.8	8.9	6.4; 11.8	9.0	6.4; 11.9	8.9	6.4;11.8	9.0	6.4;11.8	8.9	6.4;11.8	8.9	6.4;11.8	6.0	3.9;8.4
*pi*1	5.6	2.4; 9.8	5.8	2.6; 10.0	5.6	2.4; 9.8	6.7	2.9; 12.3	5.9	2.6; 10.1	5.9	2.6; 10.2	5.8	2.6; 10.0	5.8	2.6; 10.1	5.7	2.6; 9.9
*pi*2	5.4	2.3; 9.7	5.8	2.6; 10.2	5.3	2.2; 9.7	7.5	3.0; 15.9	5.8	2.6; 10.4	5.9	2.6; 10.5	5.8	2.6; 10.1	5.8	2.6; 10.3	5.7	2.6; 10.1
*pi*3	4.9	3.5; 6.6	5.0	3.5; 6.7	4.9	3.5; 6.6	5.4	3.7; 7.9	5.0	3.5; 6.8	5.1	3.5; 6.8	5.0	3.5; 6.7	5.0	3.5; 6.7	5.0	3.5; 6.7
*pi*4	10.2	8.0; 12.5	10.2	8.0; 12.6	10.1	7.9;12.5	10.5	8.2; 13.1	10.2	7.9;12.6	10.3	7.9; 12.8	10.2	7.9; 12.6	10.2	7.9; 12.6	10.2	7.9; 12.6

Model 1: non informative prior, β (1, 1), used for prevalence in the market populations (*pi*1 and *pi*2); Model 2: non informative prior β (1, 1) used for prevalence in the household populations (*pi*3 and *pi*4)

Model 3: non informative prior β (1, 1) used for prevalence in the all the populations Model 4: highly informative prior β (15.03, 2.56) used for Se bELISA

Model 5: non informative priori β (1, 1) used for Sp bELISA Model 6: non informative prior β (1, 1) used for Se HI Model 7: non informative priori β (1, 1) used for Sp HI

Model 8: non informative prior β (1, 1) used for Se iELISA Model 9: non informative prior β (1, 1) used for Sp iELISA

*pi*1 = Prevalence wet season market; *pi*2 = Prevalence dry season market; *pi*3 = Prevalence wet season household; *pi*4 = Prevalence dry season household

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6.7 Discussion

This study evaluated the performance of three serological tests (bELISA, iELISA and HI test) for the detection of NDV antibodies in unvaccinated village chickens under field conditions, in the absence of 'gold standard' test, using a Bayesian framework. A modification of the two population approach was used because sampling was done during two different seasons, expected to produce a variation in prevalence. The prevalences for the household and market datasets were estimated separately, although data were fitted in a single model. Disease status is ideally judged by the presence or absence of the disease causing pathogen, but since we used serological tests, the presence of antibodies was considered as NDV infection or exposure. However, as the period of persistence of NDV antibodies in chickens has not been clearly established, it would be difficult to determine the stage of the disease or the actual presence of infection.

The analyses revealed that bELISA performed better than the HI test with respect to its *Se* and *Sp* (Table 6.3). Both exhibited good *Se* and *Sp* with agreement in the household dataset of Kappa = 70.4%, similar to what has been reported in vaccinated flocks (Czifra *et al*., 1998). The *Se* of bELISA and HI test were comparable to those reported during validation (Czifra *et al.*, 1996). The *Se* of iELISA was also higher than that of HI and fairly similar to that of bELISA, however the *Sp* of iELISA was much lower than the *Sp* of both bELISA and HI. The *Se* and *Sp* of a serological test are affected by several factors, including the conditions under which the test is performed, the stage or duration of the infection, the presence of cross reactions from organisms of similar antigenic structure and cut-off values used to interpret tests (Greiner and Gardner, 2000). Whether the testing is performed on natural infected or experimental infected animal may also play a role, since it has been established that, for avian influenza, HI performs more accurately in naturally infected than in experimentally infected birds (Comin *et al*., 2012). The sensitivity of HI is affected by the cut-off values used ($4\log_2$), but lower cut-off values are considered prone to non-specific reactions (OIE, 2008b). A higher cut-off value ($>4\log_2$) than this, would increase the reproducibility of results but at the expense of decreased *Se*. Moreover, the *Se* of the HI test might be more dependent on the antigenic differences of virus strains, whereas the *Se* of the bELISA is less variable in response to strain variation (Czifra *et al.,* 1996). The different test methods of the HI test and the ELISA mean that antibodies to different antigenic determinants of the virus are measured

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in each assay. While the former test specifically detects antibodies directed against epitopes of the haemagglutinin neuraminidase protein of APMV-1, the ELISA detects mostly a wider spectrum of antibodies specific for different viral proteins (Czifra *et al*., 1998) unless designed to detect specific antibodies against a selected structural protein. Furthermore, it has been observed, in avian influenza diagnosis, that the performance of HI test is strongly influenced by the homology or relatedness between the reference viral antigens and the virus isolate or antibodies to be tested (Escorcia *et al*., 2010) and this may be equally valid for NDV. Blocking antibodies developed against the PMV-1 epitope, like the one used in the bELISA, are specific for the NDV infection in any host and at any age (Koch *et al.,* 1998). The onset and amount of antibody production against the different proteins of NDV might vary with the time after infection and affect test parameters and hence estimation of prevalence. A time dependent discrepancy between the HI test and ELISA has been observed for the detection of antibodies to a haemagglutinating NDV antibody in domestic geese and Muscovy ducks after immunization (Hauslaigner *et al.,* 2009). It was suggested that this phenomenon was due to different test methods: both IgG and IgM, which predominates during the first 2 weeks of a primary immune response, are able to agglutinate viral particles (Davison *et al*., 2008) and are therefore likely to be measured by the HI test. Furthermore, Hauslaigner *et al*. (2009) suggested that the differences in the antibody pattern leads to a greater number of false-negative results in the HI test than in the ELISA, more specifically at later time points after virus contact, and that the ELISA provides a higher detection level than the HI test, which is advantageous in surveillance programmes. The results of our study support this. Since HI is commonly used in NDV antibody diagnosis its performance has rarely been questioned. However, studies have shown that the variation between the quantitative test results of different laboratories using the NDV HI test was higher (about double) compared with the variation within commercial ELISA systems (de Wit *et al*., 2007).

There was no published information available on *Se* and *Sp* of the iELISA and hence the iELISA priors were based on the manufacturer's claim of *Se* and *Sp* >95% (Laura Fournier, 2011, *personal communication*). Comparing this with the posterior estimates of the test properties the iELISA exhibited a high *Se* of 95.2%, (95 % PCI: 88.5, 99.0), but a remarkably low specificity of 8.9% (95% PCI: 6.4, 11.8) compared to the other two tests for the screening of the field sera. There could be several reasons for this. The apparently much lower specificity of iELISA on field samples might be attributed to use of inappropriate low cut-off values that categorized most field samples as positive. It may be that a different cut-

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off should be used in village chickens. The presence of other cross-reacting APMV in the field, although not investigated, is another possible explanation for the low *Sp* of the iELISA since other APMVs were observed to increase ELISA absorbance (Adair *et al*., 1989) .or there might be a back ground reaction that has increased the OD values of the sample in iELISA assay. Cross reactions between APMV-1 and other APMV subtypes, including APMV-2, APMV-3, and APMV-7, have been demonstrated in the HI test and in an ELISA involving whole virus antigen (Alexander *et al*., 1983; Nayak *et al*., 2012). It is therefore plausible that a similar phenomenon might be exhibited using the iELISA, unlike the bELISA, where specific monoclonal antibodies are used. The apparently poor *Sp* of iELISA in this study calls into question its suitability for field investigation of village chicken NDV antibody status or for surveillance in unvaccinated flocks in Ethiopia.

Two test outcomes for a given animal are likely to be correlated if both tests measure a similar biological phenomenon (Gardner *et al*., 2000). Since both ELISA and HI tests are based on the same principle, namely the detection of humoral immune responses induced by NDV, although different antigenic proteins are targeted a certain degree of dependence between test results was expected. However, conditional on disease status, i.e. presence or absence of antibodies to NDV, the serological tests evaluated in this study appeared conditionally independent, evidenced by correlation (*rho*) values both for truly diseased (*Se*) and healthy (*Sp*) animal status that clustered around zero in the posterior estimation.

The prevalence estimates of NDV antibody, based on household dataset, varied from 5.0% (95% PCI: 3.5, 6.7%) in the wet season to 10.2% (95% PCI: 8.0, 12.6%) in the dry season, while the prevalence was low (5.8%) for the market dataset and did not vary between seasons. Earlier Getachew (2009), using the same bELISA, reported an overall seroprevalence of 5.6 % (95% CI: 3.2, 8.8%), closer to the estimate in the present study, in village chickens in south west Shewa, Ethiopia. Considering the village chickens were not vaccinated, the seropositive results obtained at villages were likely to be a true reflection of field NDV seroconversion status of individual chickens. The apparent low prevalence is likely the reflection of the fact that village chickens in the study area were more affected by virulent virus with likely high mortality and few seropositive survivors. The estimate of sensitivity and specificity for the tests (bELISA and HI) obtained in the present study is considered to allow unbiased estimates of the prevalence in the study area. The prevalence estimates from the household dataset reflects the situation reported from other African

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countries, i.e. a seasonal variation in incidence of ND, which was higher during the dry season (Sa'idu *et al.*, 2006; Nwanta *et al.,* 2006; Otim *et al*., 2007).

In fact for the assumption of differing prevalence, as in the latent class analysis, a bigger difference was considered better for the analysis (Toft *et al*., 2005). But in present study since the prevalence were not very different, especially for market data, this might undermine the assumption of differing prevalence and could be regarded as a limitation. A potential source of bias was the possibility that some individual chickens may have been re-tested during the second sampling. Moreover, the tests were applied on unvaccinated chickens presumably at different stages of infection. Both ELISA and HI tests are subject to false negative results that may result from low antibody titre, because of the decline in antibody titre after natural infection associated with phase of infection (Bell and Mouloudi, 1988). Schelling *et al*. (1999), applying bELISA in Swiss backyard flocks, observed poor reproducibility and low *Sp* of the test in a very low disease prevalence situation with weakly positive samples while Gohm *et al*. (1999), checking sera from the specific pathogen free and immunized chickens, found an excellent *Se* and *Sp* for bELISA.

According to sensitivity analysis, it appeared that the models generated consistent parameter estimates and were not very sensitive to selection of priors as demonstrated by the large overlap of PCI (Table 6.5). The selected model fit the field data very well, but since informative priors were used on all the parameters it appears the posterior estimate might not escape from being influenced by this information than only dictated by the field data. The limitation in this respect is mainly associated with the paucity and/or accuracy of the available information which has been used as prior. However, from a purely Bayesian perspective, one is simply modelling uncertainty about parameters and does not expect ultimately to know the precise values unless the quality of the prior information is extremely precise (Enue *et al*., 2000). Nevertheless, difficulty of evaluating models that considers dependence between or among tests, without sufficient informative priori has been noted (Toft *et al*., 2005). Although it was not validated on extensive data sets, the approached followed in this study, combining data from different sources in one model, could produce valid estimates of test parameters across different populations. Further evaluation using data from different geographical areas, preferably with differing prevalence, would contribute to better diagnostic test parameter estimates since it has been shown that estimates for the diagnostic *Se* and *Sp* may vary among populations and/or subpopulations of animals,

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conditional on the distribution of influential covariates or even the sampling strategy (Greiner and Gardner, 2000).

In conclusion, serological tests are a useful tool to assess NDV infection status of a population, indirectly based on past exposure, particularly where other diagnostic techniques such as RT-PCR are not readily available. Our estimates of *Se* and *Sp* of both bELISA and HI show that they can be used for diagnosis and effective surveillance of NDV antibodies in unvaccinated chickens under field conditions similar to those in our study. Given ease of automation, bELISA may be preferred. On the other hand, given its high specificity, HI may be used as a confirmatory test if an ELISA of high *Se* and low *Sp* like iELISA is used for screening. This is especially helpful to assess situations where individual chicken NDV antibody is generally considered low, following serial test interpretations to improve the specificity and the positive predictive values. The use of local virus as antigen and locally-produced antiserum in HI should be investigated. The presence of other potentially cross-reacting APMVs in village chickens also requires further investigation. To avoid biased results or erroneous inferences, serological tests should ideally be evaluated in a population similar to the one in which they will be used. In the presence of reasonable prior information, a Bayesian approach can provide improved inference on test performance.

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CHAPTER SEVEN

POULTRY MARKET CONTACT NETWORKS IN ETHIOPIA – IMPLICATIONS FOR DISEASE SPREAD AND SURVEILLANCE

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7.1 Abstract

The spread of poultry diseases may be influenced by movements via markets, and movement data represent a valuable source of information in this respect. Therefore, this study was conducted to understand contact structures of poultry markets in the mid-Rift Valley and surrounding areas of Ethiopia during two periods in 2010 using social network analysis. Period one was around Ethiopian Christmas and period two during the two month Ethiopian Orthodox fasting season. During each period, data on weekly poultry trade were gathered via structured questionnaires from 208 (period one) and 109 (period two) traders. The networks exhibited scale-free characteristics and weak connectivity during both periods, with a density of 3.9% and 4.5%, for periods one and two, respectively. A few markets emerged as more central in the networks, in terms of their betweenness (Meki, Arsinegele, Shashemane, Ziway and Bulbula) and 'out-degree' (Meki, Qoshe, Alemtena) during both periods, and additionally Doni (period one) and Bulbula (period two), when evaluated along with chicken trade volume and frequency of trader movement. These markets could be considered for targeted surveillance, and markets with high in-degree (Akaki, Shashemene, Debrezeit and Nazreth) for surveillance that involves traders through creation of awareness. While there was slight variation in the out-degree centrality of markets between periods there was considerable variation in out-going trading events (number of traders and quantity of chickens moved) among markets. The study provided useful insight into the contact pattern of live poultry market channel, which may help to trace the potential spread paths for diseases if an infection would start at a certain market and assist in the selection of appropriate surveillance approaches for highly pathogenic avian disease. Further studies, based on regular capturing of data in a wider part of the country, would improve the understanding of the poultry contact network and contribute to the identification of an appropriate surveillance and control strategy for infectious poultry disease in the country.

Keywords: poultry, market, contact network, Ethiopia

7.2 Introduction

In Ethiopia over 97% of the 49 million head of poultry are kept as indigenous chickens in a village production system (CSA, 2011) and serve as food and source of income for a high proportion of rural households (Wilson, 2010). Large scale commercial production systems

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represent less than 2% of the national poultry population. Rural chickens contribute over 98% of the national egg and chicken meat production (Melesse, 2000; Tadelle *et al*., 2002). Most consumers in Ethiopia prefer to buy local chicken from village producers or traders, since they are considered to be tastier and better suited for preparation of the traditional chicken sauce, locally called '*doro wot'* (Aklilu *et al*., 2007). Hence, the bulk of live poultry for marketing comes from the village production system where chickens are directly supplied by producer-sellers (farmers) to live bird markets, hereafter referred to simply as markets, and sold either directly to consumers or to intermediaries (those who buy from farmers and sell to traders, acting as middlemen in the same market) or to traders who sell them at other markets. Religious festivals and other social events periodically shift local demand, prices and quantity of live poultry at different markets (Akililu *et al*., 2007; Halima *et al*., 2007, Moges *et al*., 2010). Traders are key players in the marketing channel, moving chickens from diverse sources between markets (Moges *et al*., 2010). But their pattern of movement during different periods (peak or low marketing periods) is not well established, except in a study which showed the role of a few markets in risk-based surveillance (Vallée *et al*., 2012).

Several infectious and highly contagious poultry diseases are known to exist in Ethiopia, both in rural and commercial settings (Lobago *et al*., 2004; Tadesse *et al*., 2004; Cardinale, 2005; Halima *et al*., 2007; Chanie *et al*., 2009; Mazengia *et al.,* 2009; Chaka *et al*., 2012a). Within the country, infectious poultry diseases may spread with chicken trade movements, such as buying, selling and exchanging of poultry. An outbreak of infectious poultry disease such as Newcastle disease (ND) in village chickens can lead to severe losses, sometimes even 100% mortality in the flock (Alexander *et al*., 2004). In the face of such outbreaks, farmers choose to go to markets and sell the animals to get salvage values rather than reporting cases to the authorities (Tadelle *et al*., 2003b). This could make disease detection and investigation, and enforcement of control measures very difficult or inefficient. Furthermore, the Ethiopian animal disease surveillance and reporting system is still not well organized, with technical and budgetary limitations at various levels. Understanding of live poultry trade patterns among or between markets could provide useful information on the potential spread of infectious poultry diseases via trade movements and hence help in the design of an appropriate surveillance strategy. It also has an implication in disease control by facilitating application of policies and strategies to manage risk and prevent spread of highly infectious poultry diseases such as ND and highly pathogenic avian influenza (HPAI).

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The role of animal movement in disease spread has been demonstrated using network studies (Stegman *et al*., 2004; Ortiz-Pelaez *et al*., 2006; Dent *et al*., 2008; Dube *et al*., 2011), a method which has been used extensively in human epidemiology for predicting the spread of infectious diseases such as severe acute respiratory syndrome (SARS) and acquired immune deficiency syndrome (AIDS) (Bell *et al*., 1999; Colizza *et al*., 2007). Network analysis concepts, terminology and use in preventive veterinary medicine have been reviewed (Dube *et al*., 2009; Martinez-Lopez *et al*., 2009a). Social network analysis is a useful technique to identify individuals, populations, livestock operations (farms, markets) and regions that are important in terms of risk for disease introduction, maintenance and dissemination and could be identified and targeted for surveillance and/or disease control strategies (Christley *et al*., 2005a; Dube *et al*., 2009; Martinez-Lopez *et al*., 2009b). Selection of surveillance targets, in a more systematic and focused way was suggested as more cost-effective when resources are limited (Stark *et al*., 2006; Cannon, 2009). Network analysis would help also to understand the potential route for the spread of epidemics (Dent *et al*., 2008). The method has been used for the study of poultry movement in Cambodia (Van Kerkhove *et al*., 2009), Vietnam (Soares-Magalhães *et al*., 2010), the United Kingdom (Dent *et al*., 2008) and the Oromia region of Ethiopia (Vallée *et al*., 2012). Several types of network have been described in livestock movement and related areas: scale-free, in which a few holdings have vast majority of contact or movements (Christley *et al*., 2005b; Bigras-Poulin *et al*., 2006), small-world, which has short path with high clustering (Christley *et al*., 2005b), random, which has short path with low clustering and scale-free (Kiss *et al*., 2006a), and scale free with small world properties (Natale *et al*., 2009). The type of network can influence the rate of spread of diseases. For instance, in a scale-free network, where the farms are highly connected, disease can spread faster compared to other type of networks of similar size such as random networks (Kiss *et al*., 2006b).

Network data can be acquired from an existing livestock movement database that facilitates tracing of animals (Dube *et al*., 2009). However, in the absence of such a database, it can be generated from a questionnaire administered to producers or traders (Christensen *et al*., 2008). The situation in Ethiopia is represented by the latter scenario, with no recorded data kept for any kind of animal movement. The objective of this study was to describe patterns of live poultry trade and to apply network analysis methodologies to understand the contact network of poultry markets in the mid-Rift Valley and surrounding areas in Ethiopia during the two different periods in 2010. A further objective was to evaluate the potential role of

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markets in disease spread and to inform the design of surveillance approaches for infectious poultry diseases at markets.

7.3 Materials and methods

7.3.1 Study area

The study was implemented mainly in markets in the mid-Rift Valley areas of Eastern Shewa and West Arsi zones of the Oromia region and adjacent markets in Southern Nations, Nationalities and People's Region (SNNPR) (Figure 7.2) and was complementary to the epidemiological, virological and serological studies on ND presented in this thesis. The area has moderate poultry density, with several markets both in rural and urban centres.

7.3.2 Selection of poultry markets

Two cross-sectional surveys were conducted in markets in the South East Arsi, Eastern Shewa and eastern parts of Silte zones of Ethiopia to evaluate the weekly trading practices during two different periods of the year 2010, representing the peak for poultry movement (period one, around Ethiopian Christmas, in January) and low movement (period two, during the two month Ethiopian Orthodox fasting season, characterized by abstention from all animal products, in March) in order to quantify the spatial and seasonal variability of the contact structure. The markets, identified from a complete list of markets (n = 39) acquired from local agricultural office experts, were visited within a period of 2-3 weeks to capture representative data for each of the selected periods. Three markets in neighbouring Silte zone were included in the initial lists of markets for visit on market day based on preliminary observation in which they were found directly linked to markets in mid-Rift Valley area having an epidemiological interest.

7.3.3 Questionnaire data collection

A piloted, structured questionnaire (Appendix D) was used and all traders present at each market, on the day of the visit, were interviewed by the principal investigator and five previously trained veterinary students. Traders were defined as people who buy chickens and eggs at a market and sell them in another market(s). Data collection was done by visit to markets on their market day or based on traders visit information. Respondents were asked

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to provide information on their trading characteristics and practices: frequency (regular, occasional), type of poultry products involved (live chickens, eggs or both), purpose of visit to that specific market, biosecurity measures practiced at home with poultry and where they had sourced and sold poultry during the previous one week period. A one week window period was used to minimize recall bias and capture representative information for that specific period. Markets were visited once for interview during each period. In addition, each visited market was described in terms of the number of farmers and traders present on the day of the visit and its geographic coordinates.

7.3.4 Network characteristics

A description of the each of the network characteristics used in this study was adapted from Christlay *et al*. (2005b), Dube *et al*. (2009) and Lockhart *et al*. (2010) with slight modification to the conditions of the present study and is indicated in Table 7.1. For the purposes of this study contact was defined as a link between markets via trader(s) movement or visit.

Table 7.1 Description of network measures used in this chapter.

Parameters	Descriptions
Node	The unit of interest in network analysis i.e. live poultry markets in the present study.
Measures of cohesion	Measures to determine the level of connectivity in the network, such as density, clustering coefficient and geodesic distance.
Density	The proportion of all possible contacts among market that is actually present in the network.
Clustering coefficient	The average probability of an individual market being directly connected to another market in the network. The possible maximum value of 1 indicates that every market is directly connected to all other markets in the network.
Geodesic distance	The shortest path length between two markets.
Diameter	The longest geodesic between any pair of market in the network.

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Measures of centrality	Measures to identify the importance and role of individual nodes (markets) in the network such as: degree distributions and betweenness.
In-degree	The number of contact (links) to a market (i.e. on-market movement) from other market (s).
Out-degree	The number of contact (links) from a market (i.e. off-market movement) to other market(s).
In/out-degree centralization	The deviation of the largest values of in/out-degree from the value computed for all other nodes (markets) in the network.
Betweenness centrality	The share of times that a market *i* needs a market *k* (whose centrality is being measured) in order to reach a market *j* via the shortest path. The betweenness of a market *i* is defined to be the proportion of shortest paths between pairs of markets (excluding market *i*) in a network that pass through *i*.
Path length	The number of contacts on the shortest route between a pair of markets in the network.
Shortest-path betweenness	The proportion of times a market lies on the shortest path between other markets.
Flow betweenness	The amount of flow through market *i* when the maximum flow is transmitted from *s* to *t*, averaged over all *s* and *t*.
Normalised betweenness	The frequency with which a market falls between pairs of other markets on the path connecting them. It is obtained by dividing betweenness by its maximum value.
Normalized degree	Obtained by dividing simple in/out-degree by the maximum degree possible, which is usually N-1, yielding measure ranging from 0 to 1, can be expressed as percentage.

7.3.5 Data analysis

The questionnaire data were entered in to a relational database (Access 2007; Microsoft Corporation, Redmond, WA, U.S.A.), and later transferred to excel spread sheets (Excel 2007; Microsoft Corporation, Redmond, WA, U.S.A.). Duplicate information from traders

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interviewed on multiple occasions was cross-checked and removed. Data on the trade characteristics and markets were summarized and presented descriptively.

Social network analysis methods were used; directed valued networks were drawn using R (R Foundation for Statistical Computing, Vienna, Austria, http://www.R-project.org) and parameters were computed in UCINET (Software for Social Network Analysis, Harvard, MA: Analytic Technologies) and in R. In the networks markets represented the nodes, trader movements were the links between the nodes, and the volume of the chickens traded among the markets was the value of the link or tie. For data on source-destination pairs the quantity of chickens moved via traders in a week was aggregated and an adjacency matrix was built for each of the two periods (networks). The number of traders at markets was compared between the two periods using a Wilcoxon signed-rank test.

Analysis of each network in terms of size (number of nodes, number of directed links, and diameter), centrality measures (degree, betweenness and average geodesic distance) and network cohesion/connectedness (density) was conducted. For parameter analysis, non-valued networks were considered focusing on the presence or absence of a contact. To compare characteristics between the two study periods, those markets and links which were not present during period two were removed from the network of period one. Then bootstrap *t*-test was used to test the difference in the probabilities of links (or density) between the two periods. The quadratic assignment procedure (QAP) was used to calculate the correlation between the two binarized matrices by generating other matrices with value of 1 (where there is overlap of the two matrices) or 0 otherwise, in a simple matching (proportion of matched presence/absence of link) (Simpson, 2001; Hanneman and Riddles, 2005).

To understand the position and influence of all the markets during each study period, they were evaluated by combining their centrality measures (in-degree, out-degree and betweenness) along with their trading events (number of traders involved) and quantity of chickens traded between two markets and then broadly categorized as either “sending” or “receiving” markets, while a market could exhibit both characteristics. In “sending” category market’s out-degree (neighbouring contact), betweenness (normalized), number of traders moving chickens from individual market and quantity of chicken moved were considered while for “receiving” markets in-degree, betweenness, number of traders bringing in chicken to a specific market and quantity brought-to were considered.

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Using the coordinate data, each market location in the network was visualized in Arcview 3.2 (ESRI Systems, Redlands, CA, U.S.A) and the network in R in order to visually assess the contact structure. The geocodes and details of all the markets included in the study are indicated in Appendix E.

7.4 Results

7.4.1 Trader characteristics and poultry trade movement

Demographic information was obtained for 73.8% (31/42) of the markets on the initial lists visited directly on their market days (ETM-1 to ETM-31) and/or interviews of the traders. Additionally, data on the other markets (n = 13), not on the initial market lists, were captured indirectly from the information provided by the interviewed traders and included in the network. Data from 244 and 129 trader interviews were collected at the different market places during study period one and two, respectively. Excluding those traders interviewed at multiple market places the actual numbers of traders were 208 and 108, respectively, with 15% (32/208) and 17% (18/108) of the traders having been interviewed more than once in different market places during periods one and two, respectively. The median number of traders per market was 6 (range 2-24) and 4 (0-11), for period one and period two, respectively. There were significantly more traders per market during period one than period two ($P < 0.0001$). Of the 208 traders interviewed during the Christmas period, 56 (27%) were occasional traders who exercised chicken and chicken product trading activities during the Christmas holiday, because of increased demand for poultry products resulting in increased prices and higher profit. More male traders (87.9% and 80.6% for periods one and two, respectively) were observed at the markets during our visits (Table 7.2). Rural markets were mostly attended by farmers while in bigger urban markets traders predominated. Traders visited up to four markets to buy, but sold their products in three or fewer market places.

Based on our observations, chickens were directly supplied to markets situated in rural areas closer to villages by farmers (producer sellers) and then carried from market to market via chicken traders. In some places there were involvements of intermediaries (middlemen) who buy from farmers and sell to traders in the same market.

During period one, 64% (134/208) of traders reported that they brought live purchased or unsold chickens back to their homes, with poor biosecurity measures at home, before taking

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them again to market for sale, usually l-7 days later. Of these, 10% (14/134) reported keeping backyard poultry at their home. Poultry were usually moved on trucks in batches from the source markets to the selling markets.

Table 7.2 Number of traders and trader characteristics during the two periods of year 2010 at poultry markets (n = 31) in mid-Rift valley area of Ethiopia.

Description		Number of traders interviewed (%)	
		Period one[a]	Period two[b]
Traders		208	108
Sex	M	183 (87.9)	87 (80.6)
	F	25 (12.0)	21 (19.4)
Frequency of activity	Regular[c]	141 (67.8)	108 (100.0)
	Irregular[d]	11 (5.3)	-
	Occasional[e]	56 (26.9)	-
Product traded	Chicken	147 (70.7)	73 (67.6)
	Eggs	10 (4.8)	7 (6.5)
	Both	51 (24.5)	28 (25.9)

[a] Christmas period.

[b] Fasting period.

[c] Poultry trade was a frequent activity as main livelihood means.

[d] Traded infrequently along with other items (side business).

[e] Only traded during holidays.

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7.4.2 Network data analysis

7.4.2.1 Poultry movement

The geographic location of the markets for the poultry movement for the two periods is shown in Figure 7.1. Using data collected during the two periods, valued and directed networks were constructed with 55 and 43 unique nodes and, 115 and 82 links during periods one and two, respectively (Table 7.3). Figure 7.2a and 7.2b show contact network for the poultry movement among markets in the study area during periods one and two, respectively.

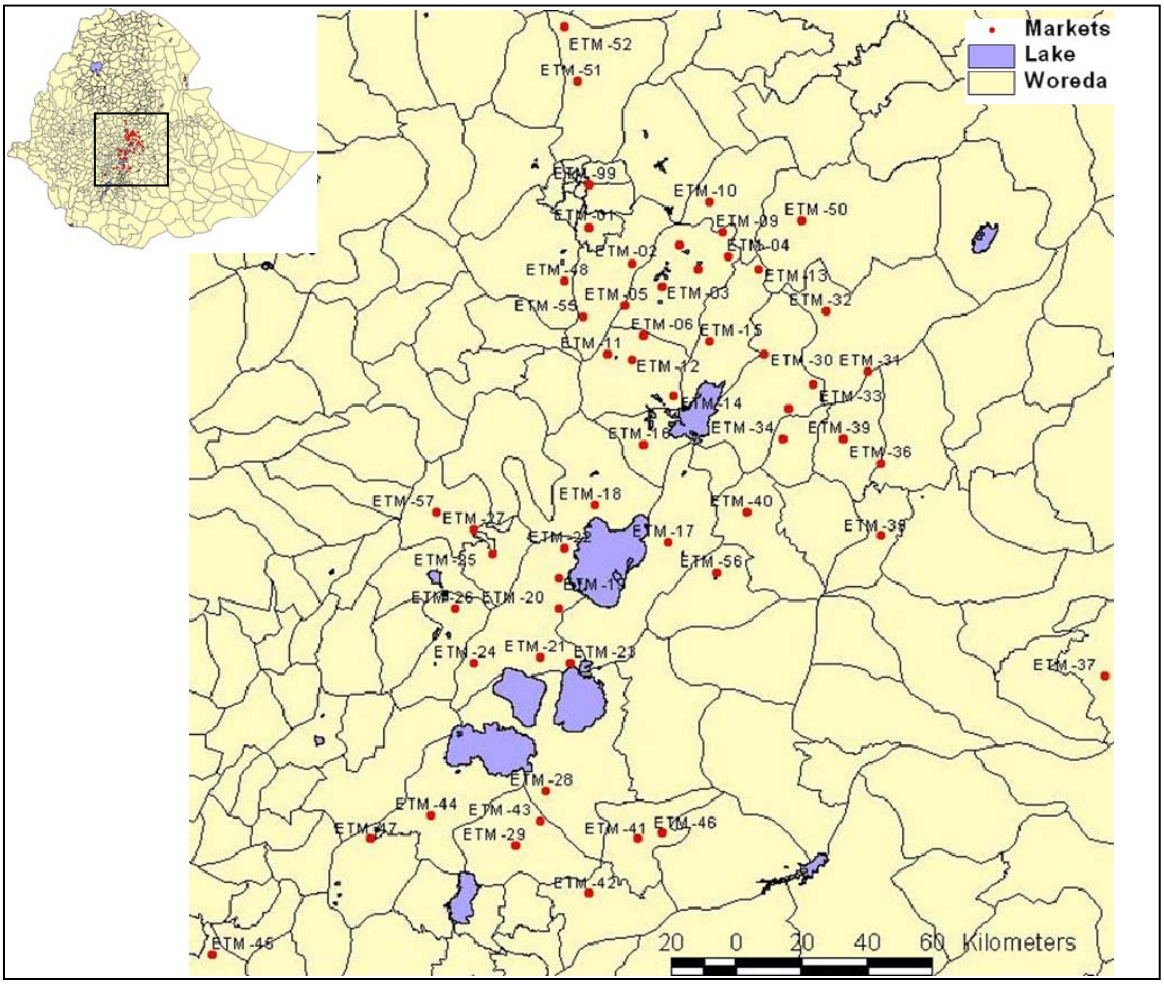

Figure 7.1 Map of the mid-Rift Valley area of Ethiopia showing the point locations of the markets surveyed directly (ETM-1 to ETM-31) and indirectly (others) in 2010.

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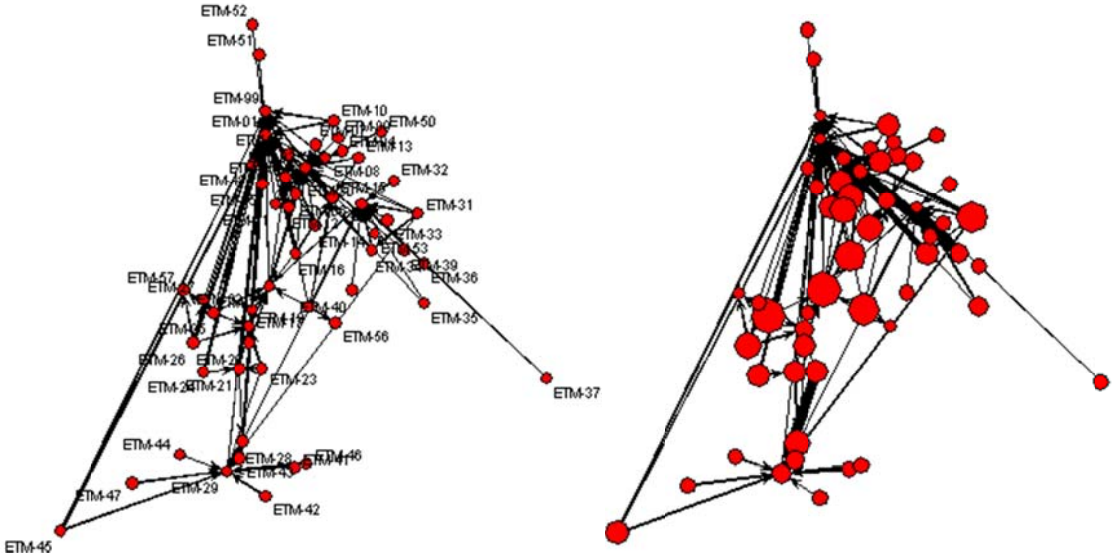
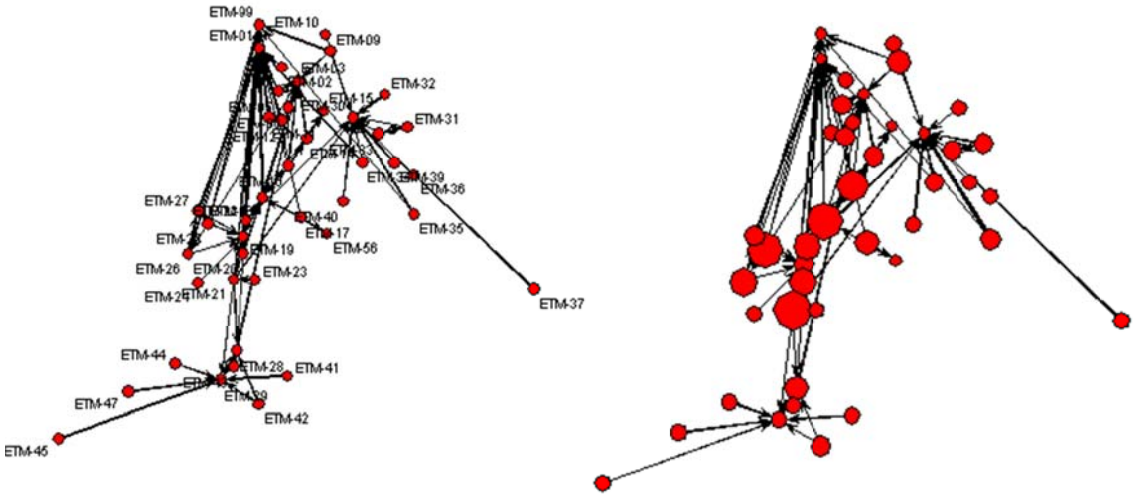


a) Period one



b) Period two

Figure 7.2 Graphical representations of contact networks for live bird movements during period one (a) and period two (b) of year 2010 in mid-Rift valley area of Ethiopia. The graph on the left shows the contact structures; on the right, node size indicates the out-degree of the markets, the arrows indicate direction and the thickness of the link indicates the volume of chickens moved between two markets.

The width of the links/ties indicates the amount of chickens moved by traders between two markets and the size of the nodes corresponds to their out-degree (number of off-market contacts), indicating their influence on the volume of chickens flowing through the network.

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Twelve markets were identified as not connected to the rest of the markets or absent in the network during period two, compared to period one. The weekly number of chickens traded through the network for the two periods was 16,182 and 5,067 respectively, and the mean link value was 140 (6-1612) and 62 (2-269) chickens, respectively.

Table 7.3 Description of network parameters for the poultry network in mid-Rift valley area of Ethiopia during two periods of the year 2010 (non-valued network).

Parameters	Period one		Period two
Size (number of nodes)	55	43*	43
Number of links	115	98	82
Density	0.039	0.054	0.045
Average geodesic distance	4.8	4.7	4.7
Diameter	4	4	4
Mean degree (in/out)	2.09	2.28	1.91
Mean normalized in/out-degree (%)	3.87	5.43	4.54
Mean betweenness	2.15	2.42	2.74
Clustering coefficient	0.38	0.22	0.54
Centralization indices (in-degree) (%)	41.32	40.76	27.04
Centralization indices (out-degree) (%)	7.37	9.07	14.42

* after 12 markets were removed (those not found in the network during period two)

7.4.2.2 Centrality measures of the networks

The descriptive network parameters (cohesion, centrality measures) for the two periods are presented in the Table 7.3. In period two a decrease in movements was observed, resulting in a network with fewer links (82) among the markets (Table 7.3). Considering only the network with 43 markets present during both seasons the number of the link was slightly higher for period one (98). The density of both networks showed low connectivity, with the

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proportion of possible ties present as 0.039 (115/2970) and 0.045 (82/1086). About 69% (38/55) and 58% (25/43) of the markets were just sender markets, while 9% (5/55) and 14% (6/43) of them were just receiver markets for periods one and period two, respectively.

The network exhibited a typical scale-free character with very low average in- and out-degree. The degree distribution for both periods is shown in Figure 7.3a and b and reflects the scale-free nature of the contact network. In the network 20 (36%) and 17 (40%) markets had a single connection to another market while 29 (53%) and 21 (49%) of the markets had more than two connections during periods 1 and 2, respectively.

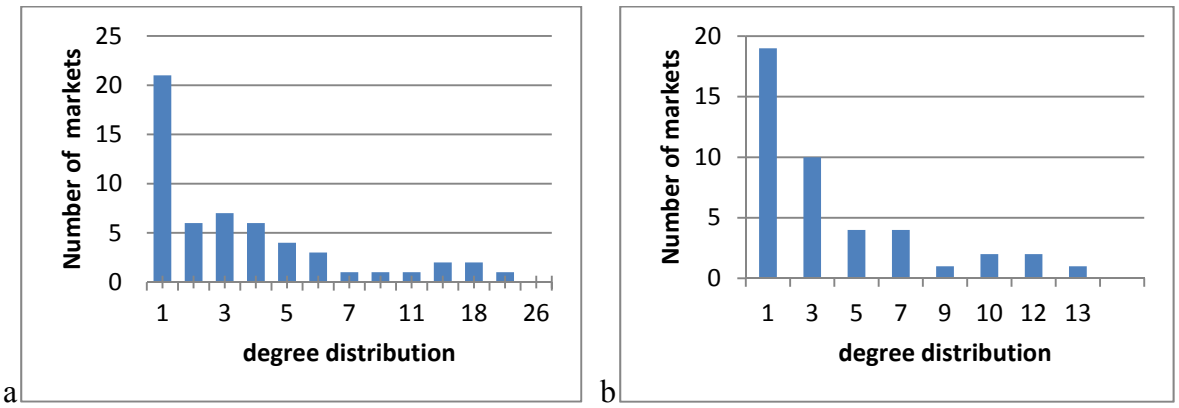

Figure 7.3 The degree distribution of non-valued market contact network for period one (a) and period two (b) of year 2010 in Ethiopia.

Considering the binarized network, centralization indices for in-degree showed higher variation (heterogeneous) during period one (41.32%) than period two (27.04%), meaning that fewer markets had high in-degree values during period one. The binarized matrices (n = 43) for the two periods showed no significant difference between their density (P = 0. 29) with 93.6% of matched presence/absence of links in QAP. Based on binarized (0 or 1) directed networks, the correlation between ‘‘market degree’’ and ‘‘market betweenness’’ were 39.7% and 55.1% for periods one and period two, indicating that most nodes had different profiles for the two centrality measures.

Tables 7.4 and 7.5 show the sorted and then ranked position of each markets (the first ten) based on combination of factors: centrality measures (out-degree or in-degree and betweenness), trading events (number of traders involved in moving chickens off-market or in-market per week) and quantity of chickens moved off-market or in–market per week for period one and two, respectively. The ranking of the totality of the live bird markets with the

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above criteria is shown in Appendix F. Accordingly, for period one, Meki, Qoshe, Alemtena and Doni were the most influential "sender markets" while Bulbula, Meki, Qoshe, and Alemtena were the most influential for period two. Interestingly, some rural markets such as Doni and Abura were found better linked to other markets (out-degree) while, while market such Tora, although linked to fewer markets (out-degree), it had high frequency of traders visit that involved in moving-out considerable quantity of chickens from that area.

Table 7.4 Sender markets: markets rankings (n = 10) based on their out-degree, betweenness and chicken trading movements (off-market) for the year of 2010 in mid-Rift valley area of Ethiopia.

Period one					Period two				
Market name (ID)	O-D	NB	TE	QC	Market name (ID)	O-D	NB	TE	QC
Meki (ETM-18)	6 (11.11)	1.16	33	3427	Bulbula (ETM-21)	7 (16.67)	0.64	13	494
Qoshe (ETM-25)	6 (11.11)	0	16	434	Meki (ETM-18)	6 (14.29)	2.60	13	377
AlemTena (ETM-16)	5 (9.26)	0	21	1752	Qoshe (ETM-25)	6 (14.29)	0	12	317
Doni (ETM-31)	5 (9.26)	0	13	424	AlemTena (ETM-16)	5 (11.91)	0.64	6	125
Abura (ETM-17)	5 (9.26)	0	7	174	Adamitulu (ETM-20)	4 (9.52)	0.05	8	351
Arsinegele (ETM-28)	4 (7.41)	0.76	6	240	Tora (ETM-26)	4 (9.52)	0	14	795
Koka (ETM-14)	4 (7.41)	0	13	405	Abosa (ETM-22)	4 (9.52)	0	7	73
Bekajo (ETM-06)	4 (7.41)	0	13	315	Arsinegele (ETM-28)	3 (7.14)	0.93	6	255
Tora (ETM-26)	4 (7.41)	0	10	301	Abura (ETM-17)	3 (7.14)	0	6	238
Adulala (ETM-12)	4 (7.41)	0	9	504	Tuludimitu (ETM-09)	3 (7.14)	0	4	163

O-D: Out-degree (normalized out-degree percentage).

NB: Normalized betweenness.

TE: Number of trading events (traders involved in moving chickens off-markets per week).

QC: Quantity of chickens moved off-market per week.

The position of "receiver markets" for both periods appeared more or less consistent, with Akaki, Nazreth, Debrezeit and Shashmene, all markets in big urban centres, at the top of the list (Table 7.5). However, the largest number of live chickens went from several markets

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identified in the network to the capital, Addis Ababa. Meki, Ziway, Arsinegele and Shashemene were markets with high betweenness during both periods. In general, there was more variability between periods for the market positions amongst "sending markets" than amongst "receiving markets".

Table 7.5 Receiver markets: markets rankings (n = 10) based on their in-degree, betweenness and chicken trading movements (on-market) for the year 2010 in mid-Rift valley area of Ethiopia.

Period one					Period two				
Market name (ID)	O-D	NB	TE	QC	Market name (ID)	O-D	NB	TE	QC
Akaki (ETM-01)	24 (44.44)	0.00	57	4436	Nazreth (ETM-30)	13 (30.95)	0.00	41	784
Addis Ababa (ETM-99)	18 (33.33)	0.00	64	5106	Akaki (ETM-01)	12 (28.57)	0.00	21	859
Debrezeit (ETM-03)	17 (31.48)	0.19	59	1805	Addis Ababa (ETM-99)	10 (23.81)	0.00	16	979
Nazreth (ETM-30)	11 (20.37)	0.00	47	1495	Debrezeit (ETM-03)	9 (21.43)	0.00	16	406
Shashemene (ETM-29)	10 (18.52)	0.91	33	1252	Ziway (ETM-19)	8 (19.05)	1.30	23	711
Meki (ETM-18)	6 (11.11)	1.16	11	344	Shashemene (ETM-29)	7 (16.67)	0.69	17	577
Ziway (ETM-19)	6 (11.11)	0.51	31	669	Meki (ETM-18)	6 (14.29)	2.60	13	272
Mojo (ETM-15)	4 (7.41)	0.08	8	219	Arsinegele (ETM-28)	4 (9.52)	0.93	4	85
Dukem (ETM-02)	4 (7.41)	0.02	8	155	Mojo (ETM-15)	3 (7.14)	0.00	9	75
Arsinegele (ETM-28)	3 (7.41)	0.76	11	272	Enseno (ETM-27)	2 (4.76)	0.00	6	110

O-D: Out-degree (normalized out-degree percentage).

NB: Normalized betweenness.

TE: Number of trading events (traders involved in moving chickens off-markets per week).

QC: Quantity of chickens moved off-market per week.

7.5 Discussion

All live poultry for trade found at the visited markets originated from village-based production, the dominant production system in Ethiopia (CSA, 2011). None of the traders

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included in this study identified any commercial or semi-commercial farms as the source for the chickens that they were moving across the market contact networks. Unlike in some other countries, where traders visit villages or communes to collect poultry (Van Kerkhove *et al*., 2009; Soares-Magalhães *et al*., 2010), chickens were supplied purely by farmer sellers directly from villages to nearby markets.

The study found that approximately twice as many traders were present and were interviewed and three times as many chickens were traded during the high trading season of Christmas compared to the low trading season. This is attributed to the emergence of occasional and irregular poultry traders during the Christmas holidays when the demand and price for poultry and poultry products is relatively higher. Period two was reported as a period of low chicken sales and consumption in Ethiopia (Akililu *et al*., 2007) while chicken sales and consumption were reported to increase with the major social and religious festivals (Demeke, 2007; Akililu *et al*., 2007). Our survey, based on trader interviews and market observations, confirmed that indeed the two weeks around (before and after) the Christmas holiday was a period of intense poultry trade activity. The two week time window period set for the surveys provided a true reflection of the respective period and reliable responses from traders, reducing possible recall bias.

The poultry market chain in the study area showed a flow pattern mainly from rural producers to nearby rural markets (unidirectional) and then to urban markets where more consumers are living and where attractive prices can be obtained. The bulk of poultry movements end in large highly populated urban centres (high in-degree) such as Shashmene and Nazreth, which are attended by a higher number of traders and fewer farmer-producers. This is consistent with observation of market channels for poultry and eggs trades in Northern part of Ethiopia (Moges *et al*., 2010). It was not customary for traders to take back chickens from such large urban markets to smaller markets for selling. However, this may sometimes occur, since in the present survey a trader was found with a batch of sick chickens brought back from the "terminal" market of Akaki (ETM-01) to sell them in Dukem (ETM-02) after he had noticed that the chickens were clinically sick, and that they might not survive much longer. After sampling we confirmed that the birds were infected with ND virus (NDV). The magnitude of such insidious trading behaviour was not investigated, but the implication of such events for disease spread along the market network could be considered serious.

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The study revealed that the networks in both periods had low connectivity, with a density of 3.9% and 4.5%, respectively (Table 7.3). This might be attributed to the fact that rural markets, supplied only by farmer-producers, are seldom connected to each other but more often to bigger district or urban markets in their vicinity. Actually, during both periods, more than 55% of markets were exclusively sending out poultry to the other markets indicating a unidirectional movement pattern. A similar movement pattern has been observed in Cambodia (Van Kerkhove *et al*., 2009). Nevertheless, during period one, the network had a relatively higher number of links attributed to a higher number of traders, with the emergence in the network of a considerable number of irregular and occasional traders (Table 7.2). These traders, according to their information, usually multiply their sources of poultry, travelling further than usual to other markets, closer to rural producers with better supply and cheaper prices for chickens. Additionally, during period two, 12 markets, which were present in the contact network during period one, were not found connected to any of the markets visited.

During period two, traders reported that they mainly moved between markets close to each other, except for a few regular traders supplying consumers in larger towns such as Nazreth and Addis Ababa. The graphical representation of the geo-referenced location of markets and their associated contacts, clearly shows how some spatially distant markets were connected via market trade movements during period one (Fig 7.2a). Furthermore, it was observed that traders visit more markets, with increased frequency, and trade at longer distances during holidays, possibly increasing the risk of spreading highly pathogenic poultry diseases such as ND. It has been reported that increased risk of spreading infection is greatly associated with movement through markets that cover longer distances (Robinson and Christley, 2007), when infected and contagious animals are moved or included in the consignment (Bigras-Poulin *et al*., 2006). This is supported by the observation that NDV strains isolated from markets as far as 200 km apart were closely clustered together on phylogenetic analysis, an epidemiological links that could be attributed to markets trade movements (Chapter 5). In fact this requires further analysis, including more virus isolates, to strengthen the inference made about the potential role of market movement.

The networks from both periods exhibited scale-free characteristics where the degree distribution fit a low power distribution characterized by the absence of peak and the presence of a long tail which gave a high variance in the number of contacts per market as depicted in Figure 7.3. More specifically, there were few markets that had much contact (degree)

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compared to the majority of the other markets in the network (Albert *et al*., 1999; Li *et al*., 2005) that implies these are markets at a greater risk of being infected or transmit infection to others as established in theoretical epidemic spread model (Pastor-Satorras and Vespignani, 2001) and therefore may be important for guided surveillance and disease control procedure (Dube *et al*., 2009).

It has been proposed that out-degree score could be a useful metric for identifying enterprises (farms or industries) more likely to disperse hazard and that risk based surveillance should target enterprises with such characteristics (Lockhart *et al*., 2010). When highly infectious poultry diseases occur in the supply area of a specific market or are brought in from other markets, markets with high out-degree could serve as points for the spread of those infections. During period one, 36% (20/55) of the markets were seen to send out chickens to at least two different markets, while the corresponding value for period two was only 23% (10/43). Hence, considering poultry movement as a potential route for the transmission of highly pathogenic chicken diseases such as ND and HPAI, those markets with high out-going contacts and trader movements have higher chance to spread the disease and need to be monitored or considered for surveillance and sampling at these markets would therefore, increase surveillance sensitivity (Frössling *et al*., 2012). Accordingly, Meki, Qoshe, Alemtena, Doni and Abura markets for period one and Bulbula, Qoshe, Meki, Alemtena and Abosa for period two could be targeted for surveillance (Table 7.4). The variation in out-degree indices between periods for some markets, as observed in the present study, may imply the need to flexibly shift surveillance strategies in terms of markets to be monitored.

For the markets sending more chickens to terminal urban markets where they are mostly destined for slaughter, implications for disease spread in the village production system, is probably limited. But, considering the behaviour of consumers, with little respect for hygienic measures in the disposal of poultry waste (visceral organs after slaughtering), and the widespread presence of free-ranging backyard chickens, even in larger urban areas, there is the possibility of back contamination of backyard chickens. However, in markets with low out-degree but where producers may buy chickens for re-stocking, the presence of infected birds at the market could have significant implications for disease spread back to villages. Ziway and Arsinegele are examples of such markets, where producers also buy chickens for restocking. Although it was difficult to capture the movement from markets back to villages, the survey team did observe farmers trying to get chickens for restocking directly from

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traders or buying from one another. In addition, we also observed traders buying only female chickens with the intention of reselling them at other markets to producers for breeding or for ritual purposes. In the complementary study of household-level risk factors for ND done in the same area (Chaka *et al*., 2012c; Chapter 4), farmers used markets as a flock rebuilding source, and this practice was associated with increased odds of NDV seropositivity or increased risk of NDV exposure. The study also recorded several farmers' opinions (during village surveys) that some of the poultry disease outbreaks in villages were caused by poultry introduced from markets to a household in their village. A similar observation was noted in Kenya where markets were cited as the risk for the introduction of poultry disease (Njagi *et al.*, 2010).

The concentration of chickens from different sources in a single point was also another interesting observation. The collection and confinement during transport of chickens probably induce some degree of stress and may increase susceptibility to diseases such as ND. Additionally, the fact that traders keep poultry together in close confinement after purchase and during transportation would enable rapid transmission of disease among chickens within a batch should a single affected or incubating chicken be sold by a farmer from an outbreak area or infected flock. The potential spread of infectious poultry disease from market, depends on the likelihood of an infected chicken entering the market chain. This might be exacerbate by the behaviour of a farmer bringing sick or incubating birds to get a salvage price in the face of an outbreak in his flock (Tadelle *et al*., 2003b; Nwanta *et al*., 2006). In another complementary study, NDV was isolated or antigen detected through rRT-PCR techniques, from sick or apparently healthy chickens at several market places in our contact networks (Chaka *et al*., 2012b; Chapter 5). Virus isolation and characterization from these markets have revealed that most of the strains were virulent viruses (Chapter 5). There is therefore a chance of purchasing clinically normal village chickens that are infected with virulent NDV (Fantanilla *et al.,* 1994). This was substantiated by an observation where a clinically normal male chicken bought at one of the markets became sick after being brought to the research station and died of a disease, which was later identified by virus isolation and molecular assay, to be a virulent NDV. The risk of this is heightened during holidays when larger numbers of chickens are brought from villages, increasing the likelihood of infected birds being included in batches for trade. Sa'idu *et al*. (2006) and Nwanta *et al*. (2006) suggested that increased movement of sick and healthy chickens in anticipation of various festivals may have been responsible for the peaks of ND outbreaks in Nigeria. Additionally,

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as evidenced from the present study, chickens stocks could spend several days (up to 7 days) in the market channel, contrary to what has been reported elsewhere (Van Kerkhove *et al*., 2009), before completely sold by a trader. Of the poultry traders reported to bringing home unsold chickens 10% (14/134) breed their own backyard chickens. In a setting with poor biosecurity (Wossene, 2006), this would constitute a real risk in terms of disease transmission and possibly spread. Similar observation was noted in Vietnam where unsold poultry in some occasions introduced back to other flocks perceived as a risk for further HPAI virus transmission (Soares-Magalhães *et al.,* 2010).

The low density of links observed in the present study meaning markets are less connected among each other, perhaps imply that if an infection is detected at any one point of the contact network, control would be less challenging. However, this may not be the case, since there might be unpredictable traders and farmers behaviour in the event of an outbreak of a highly infectious and pathogenic poultry disease. Additionally, it is not known how movement distance, patterns and frequency may change in response to disease outbreaks; this could be considered in future studies.

During both periods a few markets showed high betweenness, indicating their role in controlling flow of chickens across the network. Meki, Ziway, Shashmene, and Arsinegele were markets with such characteristics. Livestock operations with high betweenness can be targeted for disease control activities once identified (Ortiz-Pelaez *et al*., 2006). In addition, node or edge removal (removal of potentially infectious contacts) could be used as risk mitigation approach. Such measures may include closure of markets or farms, or prohibition of movement. However, in Ethiopia, removal of edge (trader movement control) or closure of markets may not be feasible strategy, since there are no established rules and regulations or ways to enforce them. Hence, the alternative approach is to work with traders through the systematic creation of awareness (informed surveillance and control), to discourage them from buying chickens from affected areas and transporting them elsewhere. This is feasible because traders themselves, for economic reasons according to their report, avoid as much as possible buying sick chickens. Traders can also provide information about poultry disease conditions in other areas, based on their observation at different market places. It has been noted that considering regular traders for targeted informed surveillance was effective, since experienced traders were observed to avoid suspected disease risk areas in the case of HPAI (Soares-Magalhães *et al.,* 2010). The potential point of action for such a surveillance

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approach could be those markets with high in-going movement contact, attended by a high number of regular traders and receiving a high quantity of chickens from different sources, such as Akaki, Shasmene, Debrezeit and Nazreth, and including Ziway for period two.

The study covered markets in a limited area and might not have captured comprehensive information. The study relied on traders' information at the visited markets. Hence, few markets which were not visited, on their market day, that appeared as weakly connected, at the periphery of the networks, might have been connected to others in different geographic areas. This might have introduced bias to a certain degree on the inference made. Since the infection status of each market was not established, the probable role and risk of each market with respect to disease spread was simply inferred from their centrality measure analysis. Nevertheless, the detection of viral genomes in apparently health chickens at selected markets during molecular investigation (Chapter 3) and isolation of viruses from chickens in several markets (Chapter 5) justify their potential in influencing the spreading of the viruses. Combining the network analysis with these findings and modelling infection spread along market channels would enhance our understanding of the role of markets in disease epidemiology.

In conclusion, the study provided useful insight into the market contact pattern of poultry movements for the periods and area studied. Evaluation of markets in the network with respect to the volume of the chicken trade and the frequency (observed patterns) of the trading events, along with centrality measures, provides epidemiologically meaningful information with respect to their potential role in disease spread and for the design of surveillance and control strategies for infectious avian diseases. Further studies, based on regular capturing of data, in a wider part of the country would improve our understanding of the poultry contact network and enhance the identification of appropriate and efficient surveillance and control strategies for highly pathogenic poultry diseases in the country.

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CHAPTER EIGHT

GENERAL DISCUSSION AND CONCLUSIONS

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8.1 Introduction

The importance of village poultry in the economy of developing countries and its role in improving the nutritional status and income of many smallholder farmers has long been recognized (Soniya, 1990; Kitalyi *et al*., 1998; Gueye, 2000; Copland and Alders, 2005; Mengesha *et al*., 2008). This holds true for Ethiopia as well. The spread of highly pathogenic avian influenza (HPAI) H5N1 into the African continent during 2005/6 drew attention to the neglect of avian disease surveillance in countries such as Ethiopia. The incidence of HPAI in the neighbouring countries to Ethiopia, Sudan and Djibouti, presented an opportunity to receive investments in training and the establishment of a high standard bio-safety level laboratory (BSL-3), which later qualified as a regional reference laboratory for HPAI and Newcastle disease (ND) for eastern Africa.

The production potential of village chickens is constrained by several factors such as management, feed and disease. This thesis focused on health problems, mainly on the epidemiology of ND. The seroprevalence of ND in relation to other infectious poultry diseases was investigated, incidence of exposure to ND viruses was estimated, risk factors at household flock levels were assessed, circulating viruses were characterized by molecular assays, different serological tests were evaluated and the role of markets in disease spread and surveillance was assessed.

8.2 Infectious poultry diseases in village chickens

It is believed that the health of village chickens is affected by a range of other infectious poultry disease besides ND. This study has substantiated this by finding that village chickens at markets had been exposed to a range of other diseases such as mycoplasmosis, pasteurellosis and infectious bursal disease (IBD) (Chaka *et al*., 2012a; Chapter 2). Similar observations have been noted from other countries (Kelly *et al*., 1994; Biswas *et al*., 2005). Studies have also reported widespread incidence of fowl pox, IBD, and internal and external parasites throughout sub-Saharan Africa (Gueye, 1999; Kitalyi, 1998; Sonaiya *et al*., 1999, Moreki *et al*., 2011). Although estimation of the proportion of losses attributed to these and other diseases was beyond the scope of this study, ND undoubtedly constitutes the major health problem, because of the high mortality it can cause. Research work in other African or

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countries in south East Asia also indicated that ND is indeed the most devastating disease in village chickens (Copland and Alders, 2005).

Concurrent seropositivity for multiple pathogens does not give any indication of the sequence of occurrence of those agents in chickens examined. Nevertheless, it is obvious that the concurrent presence of antibodies to several poultry diseases highlights their potential negative effects on the production of village chickens, since co-infection probably compromises the health of chickens in a synergistic manner, as explained for some of them (Bradbury, 1984). Infection with one agent may also increase the risk of infection with others (Carpenter *et al*., 1991). Diseases such as the immunosuppressive IBD, diagnosed in commercial or multiplication farms in Ethiopia with reported high mortalities (Zeleke *et al*., 2005a; Woldemariam and Wossene, 2007) could potentially complicate infection with other pathogens in a mixed infection. In Botswana, IBD has been shown to be endemic in indigenous chickens and predispose them to other pathogens such as *Mycoplasma* spp. and ND virus (NDV) (Mushi *et al*., 2006). Dahl *et al*. (2002), after observation of concurrent infection of free range chickens with *Pasteurella multocida* and *Ascaridia galli* postulated that infection with *A. galli* increases the risk of fowl cholera outbreaks. The serological detection of mycoplasma in this study is suggestive of the clinical importance of chronic respiratory disease (CRD) caused by *Mycoplasma gallisepticum*. It has been suggested that exacerbation of the clinical signs induced by the milder strains of NDV may occur when infections by other organisms are superimposed or when adverse environmental conditions are present (OIE, 2008b).

Because of the poor laboratory diagnostic service coverage in Ethiopia, many incidents of poultry disease remain undiagnosed and the true morbidity and mortality due to infectious poultry disease is higher than reported. The paucity of information on the presence and prevalence of the other infectious diseases in village chickens may reflect a lack of resources for disease surveillance in village production systems. Previously several other diseases have been described in large commercial poultry settings in Ethiopia (Alamargot, 1987; Nasser *et al*., 2000; Lobago and Woldemeskel, 2004; Zeleke *et al*., 2005a; Woldemariam and Wossene, 2007; Chanie *et al*., 2009). With the increase in small-scale commercial poultry production, where foundation stocks are sourced from either large farms or poultry multiplication and distribution centres, there is a potential for any infectious poultry disease in those facilities to reach village chickens. This is because small-scale production is mostly practiced in peri-

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urban areas contiguous to village settings with backyard chickens in close proximity and potential indirect contact since biosecurity is not practised satisfactorily (Wossene, 2006; Pagani and Wossene, 2008). Similarly, disease conditions in village chickens could hamper small-scale commercial production. Hence, successful development of these production systems will only be achieved when investigation and control of those diseases is undertaken in a holistic manner.

8.3 Newcastle disease in household flocks

The study revealed an average flock size of 7 adult chickens per a household, similar to what has been reported in northern parts of Ethiopia (Halima *et al*., 2007). Tadelle *et al*. (2003a) reported chicken ownership to be in the range of an average of 5.8 to 9.8 adult chickens in different regions of Ethiopia. However, village poultry flock size may fluctuate substantially, even within an individual household and a given year, depending on factors such as mortality, off-take due to market selling, availability of feed and agricultural activities. The flock dynamics between two sampling periods in households in our study area is described in Chapter 3.

The cross-sectional serological investigation at household flock level showed that NDV seroprevalence generally was higher during the dry season (27.4%) than the wet season (17.4%). The observed higher flock seroprevalence during the dry season also corresponded to reduced flock size (Chapter 3), which partly can be explained by disease losses, mainly ND, as supported by farmers' reports and demonstrated by the high incidence of virus circulation. The higher odds of household-level seropositivity and incidence of NDV exposure during the dry season is consistent with an observation in Uganda (Otim *et al*., 2007) where authors observed shorter survival time during December-February (dry period) compared to the wet season, with considerably higher risk of ND outbreaks during this period.

An interesting phenomenon observed, which has epidemiological implications, was that egg hatching was mostly done during the dry season. This is to minimize losses of chicks due to cold stress, or because of the perceived high risk of spoilage of eggs or the destructive effect that chicks may have on seasonal crops cultivated in backyards (Tadelle *et al*., 2003a). This practice results in a large number of susceptible (unprotected) young birds entering village

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flocks during the dry season. Based on data from Zimbabwe for the rates of egg incubation and loss, and of mortality in chicks and growers, the turnover of birds in the flock would result in the average flock comprising 30% potentially unprotected birds within 16 weeks (Oakeley, 1998). Newcastle disease is highly transmissible in such flocks (van Boven *et al*., 2008), often resulting in high mortality rates as observed in Myanmar (Henning *et al*., 2008). Nevertheless, hatching at home vs. other sources (buying in replacement birds or receiving as gift) was associated with lower odds of seropositivity and lower risk of NDV exposure (Chapter 4). From the disease risk point of view, using hatching at home as flock re-building strategy, i.e. keeping a closed flock, is a protective measure that should be encouraged as far as ND is concerned.

The incidence of NDV exposure of household flocks ranged between 19.7% and 25.5% (Chapter 4). Incidence relates to number of new cases of a disease in a defined population within a specific period, usually expressed in relation to population at risk (Dohoo *et al*., 2003). Hence, to estimate incidence requires follow-up of cohorts, but this is usually difficult in extensive systems like in village chickens. In this study we estimated incidence of NDV exposure (virus circulation) at household flock level using a cross sectional serological survey with two sampling times, coupled with information on flock dynamics and farmers perception of suspected ND in their flocks. We preferred this approach, since placing trained technicians in each village to identify new cases and confirming them by laboratory diagnosis was unfeasible within the scope of the project. Earlier Serrao *et al*. (2012) in Timor did incidence estimates at individual bird level, purely based on serological outcomes, through longitudinal monitoring of marked birds in villages. Because of the different approaches followed it is difficult to compare incidence estimates from the two studies. Loss to follow-up of marked birds, particularly due to virulent NDV infection, would result in biased incidence estimates. Likewise in our case, since we included farmers' reports there could be some false categorization because of farmers' misdiagnosis or those households in which seroconversion occurred but titres declined during the 8 month observation period. Although our method also had its limitations, a cross-sectional survey with two sampling points may better assist in determination of incidence of a disease at household flock level, if coupled with other flock information, in situations where longitudinal follow-up is not feasible for various reasons.

Viral genome was detected in 14.2% of households during the wet season using a fusion (F) gene assay and in 24.2 % of households during the dry season using a polymerase (L) gene

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assay that targets both class I and class II viruses. At the markets sampled overall bird level prevalence was 4.9% for period one (wet season) using the F gene assay, and 38.2% and 27.6% for periods two (dry season) and three (wet season), respectively, using the L gene assay. Although, two different molecular techniques were employed and the study failed to compare the viral circulation across different seasons, a higher prevalence of NDV genome detection was observed during dry season sampling (Chapter 3). In the majority of cases, sequencing of the fusion protein cleavage site of rRT-PCR positive household and market samples confirmed the circulation of virus strains bearing amino acid motifs characteristic of virulent viruses (Lamb *et al*., 2007; OIE, 2008b). However, the low virulent strains could still play a role in ND epidemiology by inducing an antibody response, thereby providing a certain degree of protection and possibly contributing to endemic maintenance of the viruses in village flocks (Spradbrow *et al*., 1980; Samuel and Spradbrow, 1989). It has been described that chickens immunized with low-virulent live or inactivated vaccine strains, could still become infected with virulent NDV and shed virus silently without any evident clinical disease (Kapczynski and king, 2005; Miller *et al*., 2009). In Pakistan, Munir *et al*. (2012) also recovered virulent NDV from apparently healthy backyard poultry, emphasising their role in epidemiology and spread the disease. The possibilities of carrier birds (Awan *et al*., 1994) or resistance of indigenous breeds (Hassan *et al*., 2004) have been described previously. Whether this holds true for Ethiopian village chickens requires further investigation as NDV antigen, indicative of virulent virus, was detected from apparently healthy chickens (Chapter 3).

In a study of ND transmission in village chickens in the tropics, Huchzermeyer (1993) emphasized that that transmission is mainly through contact, although it has been suggested that transmission is not as fast as in commercial flocks (Awan *et al*., 1994). Household settlement patterns should have a large influence on effective contact rate between flocks. Human settlement and population density was identified as significant risk factor of HPAI in Indonesia (Leo *et al*., 2011). Transmission could also be via indirect contacts between flocks of different households. Such mechanisms include carcass and waste disposal in an open field to which chickens from other households can get access, and movement of dogs between villages carrying dead birds that may have died of an infectious disease. As a disease control measure, advice could be provided to farmers regarding hygienic disposal of dead birds and poultry wastes in a manner that limits access by chickens, other scavenging birds or dogs. Although constant mixing of chickens is a common phenomenon, farmers reported that they

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adopted several risk mitigation strategies to limit disease transmission. These were confinement of their flocks during suspected disease outbreak in their village, or sending for a while the whole flock to a place they consider disease free, with their relatives. There is also a belief that, during the rainy season, chickens that are confined until the morning dew on the grass has dried are at lower risk of contracting a disease. Although in this case there is unlikely to be a sound scientific explanation, this and other indigenous knowledge of farmers can be further explored and may yield further insights into disease transmission mechanisms.

8.4 Molecular analysis and phylogenetic studies

The viruses circulating between villages and markets in the study area clustered together in genotype VI (Czegledi *et al*., 2006) with the maximal distance between isolate in the present study of 0.054 (Chapter 5). This indicated that closely similar NDV strains were circulating between markets and villages and underscored the role of market trade movement in their spread. Since the virus strains also exhibited different amino acid motifs at their cleavage sites this justifies the need for further phylogenetic analysis to appreciate whether heterogeneity exists among all the isolates in the present study (Chapter 5). Indeed such a genetic distance between strains is sufficient to categorize virus into different sub-genotypes (Miller *et al*., 2010b). Considering the small area covered and limited number of strains isolated and analysed, this study may not be representative of the phylogenetic diversity that may exist elsewhere in Ethiopia. Thus this should be explored further, including the analysis of many more NDV isolates, possibly in light of the new method of genotyping proposed recently (Diel *et al*., 2012). In addition, although NDV circulating in different parts of Africa has a tendency to cluster regionally (Herczeg *et al*., 1999; Abolnik *et al*., 2004; Cattoli *et al*., 2010), this has not yet clearly been described for the East African region. There is therefore a need for further broader molecular epidemiological studies in the region to establish the origin of the viruses currently circulating in Ethiopian domestic chickens. To this end, local capacity building with respect to advanced molecular diagnostic tools, including sequencing, is of paramount importance.

An interesting observation of potential epidemiological importance in this study was the close genetic relationship of the strains in the present study with pigeon paramyxovirus (PPMV) strains (Fig 5.2). Although the strains in the present study appeared distinct from the PPMV-1 group, previously analysed samples from chicken at markets in Ethiopia were attributed to

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PPMV-1 (VLA laboratory report). It is possible that there might be virus transmission between domestic chickens and wild pigeons as has been documented in South Africa (Abolnik *et al*., 2008) and Turkey (Oncel *et al*., 1997). If this is a frequent phenomenon in Ethiopia it would be a considerable threat to domestic or commercial chickens, because wild birds are common and can easily access the backyard production system, and potentially act as a link between village chickens and commercial systems. This requires close monitoring and surveillance.

8.5 Serological diagnosis of Newcastle disease and test evaluation

Although several tests have been described for the diagnosis of NDV, serological screening is the most accessible to many laboratories in developing countries for disease diagnosis and surveillance. However, a test may exhibit low or poor specificity or sensitivity as has been observed with one of the commercial inhibition enzyme-linked immunosorbent assay (iELISA) with very low specificity observed (Chapter 6). The possible reasons for this have been discussed and this could lead to incorrect conclusions regarding disease status and biased estimates of prevalence. With the extensive reliance on serological diagnostic tests and the availability of several commercial ELISA kits for the detection of NDV antibodies it is imperative to evaluate commercial ELISA tests before their use on a wide scale in village chickens.

In test performance evaluation in the absence of a gold standard, most studies have followed the model script recommended by Branscum *et al*. (2005). Although we followed the same model principle, we showed that modification and adaptation of accepted model scripts could also provide reliable estimates of test parameters. The modified model versions allowed incorporation of data from different sources (market and village) and three tests, and provided plausible estimates of the parameters of the tests and of prevalence. However, these findings need to be validated on a larger dataset.

8.6 The role of markets in poultry disease spread and surveillance

Disease or seasonal cropping may periodically force farmers to reduce flock size. Therefore, although re-building flocks by buying in chickens was shown to be a risk factor for ND (Chapter 4), a certain proportion of farmer households are always likely to replace birds from markets or other sources. Tadelle *et al*. (2003a) found that about 30% of breeding females in

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the studied households, in different region of Ethiopia, had originated from markets. Therefore, the potential role of markets as infectious poultry disease sources can easily be appreciated. Markets or access to several markets were considered as risk factors for the introduction or exposure to NDV in other countries as well (Nguyen, 1992; Otim *et al*., 2007; Njagi *et al*., 2010; Rasamoelina Andriamanivo *et al*., 2012). Poultry markets have also been identified as a major risk factor contributing to the spread of HPAI in poultry (Indriani *et al*., 2010).

The analysis of market trade movement of chickens in the present study combined different network centrality measures and market traders' characteristics to understand the role and potential influence of each market in the network (Chapter 7). Most of the markets had very few links in the network, with a small number of markets acting as highly connected hubs. The direction of movements was largely dictated by the demand for and supply to larger urban markets where more consumers reside. Interestingly, in this study there was no trader movement into villages like that observed in other countries (Van Kerkhove *et al*., 2009; Soares-Magalhães *et al*., 2010) and hence the likelihood of disease spread through such means is very low, at least in our study area. However, the possibility of disease spread back to villages due to farmers buying chickens or returning with unsold chickens cannot be excluded. The fact that the network is weakly connected can be seen as an advantage as far as containment of infectious poultry disease goes, since most rural markets were connected only to nearby woreda markets in the same area and consequently with limited roles in geographic spread of a disease to several markets in the network. The epidemiological importance of those markets with high in-degree in bigger urban centres is limited with respect to infectious disease spread; they rather have greater public health significance, as chickens are destined for slaughter and consumption.

Some other socio-cultural practices (Otim *et al*., 2007) or risk mitigation strategies practiced by farmers may also contribute to disease spread. Irrespective of market demand and price, it has been observed that farmers take chickens to markets during a suspected outbreak either in their flock or their village, in order to minimize financial losses (Tadelle *et al*., 2003b). The clue to this is presence of farmers at markets with chickens of different ages, including chicks. Market observation can therefore be used as an excellent monitoring tool to indicate infectious poultry disease in villages, unless it is during the crop farming season when farmers may also sell chickens, irrespective of their ages, to minimize their destructive effect

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on crops or gardens. Monitoring and surveillance of such events can easily be achieved by involving local veterinary personnel. Hence, implementation of surveillance approaches that involve different stakeholders is paramount importance. The role of traders in surveillance and effective control of any infectious disease that starts at villages and follow market channels is essential to consider. Besides targeting surveillance on the markets that appeared influential in the network (Chapter 7), working with regular traders would enhance surveillance and control of highly pathogenic avian disease. Although the reason why traders were not encouraged to go into villages to collect chickens was not systematically investigated; the absence of such practice, in the study area, could be considered as having positive implication from the risk perspective that the risk of spreading infectious poultry disease such as ND is minimized. It was indicated that the presence of trader (s) in a village has been indicated to be associated with the risk of HPAI, at village level, being an indicator of poultry movement within villages that may contribute for the introduction and transmission of the disease (Desvaux *et al*., 2011).

Village poultry farmers are discouraged by the periodic losses caused by circulating virulent virus strains. This is not only discourages them from investing their resources on improving their poultry production and healthcare but they also refrain from reporting incidents of poultry diseases, since they do not see the benefit of doing so. This limits our understanding of the magnitude and extent of various poultry diseases present. Education and extension to encourage farmer identification of major poultry diseases would enhance the passive disease reporting system in village chicken production systems. It would also enable early diagnosis of cases and establishment of the profile of various poultry diseases, based on laboratory confirmation, and inform official veterinary services to implement appropriate control strategies. The need for enhancement of the current limited diagnostic capacity and coverage of poultry diseases in Ethiopia is pressing, and this will eventually minimize economic and social disruption. An example of the latter was a recent suspected HPAI outbreak in a breeding farm in Ethiopia that resulted in great public panic, but turned out to be a misdiagnosis and was in fact IBD.

8.7 Prospects for further research

Results of this study have elaborated several aspects of the epidemiology of ND in village chickens in Ethiopia, based on the research questions addressed. Despite its limited scope

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both in terms of area covered and subjects addressed, the various study components contribute to the understanding of ND epidemiology in village poultry. In addition, the studies described in this thesis have identified a number of factors that are important to consider as further research topics, apart from those mentioned elsewhere in this thesis.

The detection of a high proportion of viral genomes with virulent virus motifs, in apparently healthy chickens and in ND suspected cases from villages and markets, although needing to be proved through *in vivo* tests, is of great concern and justifies the need for continuous surveillance to monitor emergence of new genotypes or lineages. To this end, there is a pressing need for continuous collaboration with reference laboratories in Africa and elsewhere in the world to acquire knowledge and skills in the advanced analysis of diagnostic specimens.

Infectious bursal disease, which is an emerging threat in commercial production systems, was shown to be present in village chickens. The seroprevalence of this disease was found to be high but no appreciable mortality was observed as reported in commercial farms. The possible reasons for these could be found by investigating the circulating IBD virus strains and/or assessing indigenous chickens' susceptibility to circulating IBD virus.

Risk factor assessment was only addressed at household level. Nevertheless, as some of the potentially important risk factors were not limited to single households but likely acted at the village level, further assessment of risk factors at village level would help to enhance our knowledge and inform control measures.

In the evaluation of diagnostic ELISA kits for the detection of ND antibodies we found apparently high sensitivity but very low specificity of one commercial kit, which may have been due to cross reaction with paramyxovirus other than APMV-1. The possible existence of these other APMV strains in Ethiopian domestic chickens requires further research.

Inferences regarding the influence of poultry markets in the spread of infectious poultry disease were based on their centrality measures and other trading characteristics. However, combining the network analysis with spread simulation and modelling would further enhance our understanding of the role of markets in the dissemination of poultry diseases.

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Implementation of effective ND control programmes in developing countries reduces mortality and morbidity in village free-range chickens, leading to positive effects on production (Clarke, 2004; Awa *et al*., 2009; Rodríguez *et al*., 2011; Sodjinou *et al*., 2012). Undoubtedly, controlling ND would also give a better chance for the detection of HPAI if it is introduced. This is because since the clinical manifestation for these two diseases are very similar, the presence of ND over-shadows the suspicion for HPAI even if it is introduced to an area. Hence, controlling ND would enhance the suspicion of HPAI in the field and even draw attention of investigation laboratory to focus more on it for diagnosis immediately. Genetic improvement efforts alone are unlikely to achieve rapid results; there is therefore a need to introduce vaccination of village chickens in Ethiopia as well. Whether the conventional commercial vaccines produced in Ethiopia (Hitchner-B1, LaSota/46, and thermotolerant I-2) could provide enough protection against clinical disease, infection or shedding by strains of viruses circulating in the study area or more widely in Ethiopia was not assessed. It has recently been reported that NDV vaccines formulated to be phylogenetically closer to the outbreak or challenge virus may provide better ND control by reducing virus shedding and transmission from infected birds (Miller *et al*., 2007; 2009). Protection due to vaccination increases with vaccine titre, which may also reduce or eliminate virus shedding, even if a classical vaccine based on Lasota strain is used to immunize birds against virus of heterologous genotype, emphasising the efforts to improve vaccine delivery (Cornax *et al*., 2012); this may be worthy of further investigation.

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10. APPENDICES

Appendix A. Newcastle disease study farmer household questionnaire

Woreda(District):		*Kebelle*/PA:		Date of interview (dd/mm/yy)
Garee /Village:				
Interviewee name:		Sex: ☐ M ☐ F	HH Code	ETH ___ _____
Village Location	E=long		N=lat	

Part I: Flock size and management

Flock size (on the day of visit)

	Chicks <2mths	Grower 2-6mths	Cocks	Hen	Total
Local					
Exotic					
Hybrid					
Total					

1. Who owns chickens in the household? _____ (1=Father, 2=Mother, 3=Parents, 4=Children, 5=everyone)
2. Who is taking care of poultry mainly in the household?_____ (1=Father, 2=Mother, 3=Parents, 4=Children, 5=everyone)
3. Where did you get chickens for restocking/flock building (could be many source)? _____ (1= Buy fertile eggs; 2= gift; 3=local market; 4=commercial farms; 5= hatching at home; 6=other specify_____).
4. a. Where do chickens rest at night?_____ (a=Kitchen/store; b= In the main house; c= Separate chicken house d= Perch on trees; e=Other specify_____)
   b. Do you clean the chicken house or waste? ____ (1=Yes; 0=No)
   c. If yes, how frequently do you clean the chicken house/waste? _____ (1=Daily; 2=Weekly; 3=Monthly; 4=less than once per month).
   d. what is done with chicken waste? _____ (1= thrown in to deep garbage place; 2= buried; 3= burnt; 4= thrown away out of once compound; 5=other specify_____
5. a. Is there feed supplement provided to chickens besides scavenging? ___ (1=Yes; 0=No)
   b. If yes, mention supplementary feed type and sources _____
   c. Where is the water source for your chickens (could be many)? ____ (1=tap water; 2=borehole; 3= hand dug well; 4= river; 5= pond; 6=other _____)

Part II. Marketing of chickens and eggs

1. a. Did you sell live chicken in the eight months or since our last visit?___ (1= Yes; 0=No)
   b. If yes, where did you sell most of the chickens (could be in many places)? _____ (a=in the same village; b=in the local market, c= on road-side; d=in town (urban center).

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2. a. Did you sell eggs in the last eight month or since our last vist for sampling? ___(1= Yes; 0=No)

   b. If yes, where do you sell most of the eggs (could be in many places)? _____ (a=in the same village; b=in the local market, c= on road-side; d=in town (urban center).

   Provide market name(s) and locations_____

3. In which months of the year (possible to select/✓check months) do you sell more chicken/eggs (provide number if possible) Why?_____

Item	Onk	Sad	Mud	Amaj	G/dha	Bit	Ebl	Cam	Wax	Ado	Hag	Ful	as available
	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	June	Jul	Agu	Sep	
Chicken													
Eggs													

4. a. Did you buy live chicken in the last eight months? _____ (1=Yes; 0=No)

   b. If yes, Why? ___ (1=home consumption; 2= breeding; 3=ritual purpose; 4= other (specify) __

   c. If yes, was there quarantine practice (keeping separately) when new chicken are introduced in to the flock? _____(1=Yes; 0=No)

   d. If there is one, how long (in days)? _____

   e. Have you had unsold chicken back home since our last visit for sampling? _____ (1=Yes; 0=No)

Part III. Health and biosecurity aspects:

1. a. Did you experience chicken loss in your flock in the last eight months? _____ (1=Yes; 0=No)

   b. If yes, mention causes and number of chickens lost.

Causes of loss	Type/number of birds			Total	Common Season for[1]	Remark
	Chicks <2mths	Grower 2-6mths	Adult			
Disease						
Predator						
Theft						
Accident						
Stress						
Other(specify)						
Total						

N.B: [1]: 1=dry season; 2=short rain S; 3= long rain S 4= all

2. a. Have you experienced any disease problems in your flock in the last twelve months?_____ 1=Yes; 0=No)

   b. If yes, indicate the clinical signs/disease and age group(s) affected.

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Type of disease /Signs	Disease name/ Local name	Age groups affected *	Period of the year **	Range of signs to select from
				Discharge(nasal, eye), Discoloration of comb, Coughing , gasping, Diarrhea (bloody/greenish, yellow), Twisted neck, Paralyzed legs/wings, Swollen head, Swollen joints, Fowl pox/warts, Sudden death, etc

N.B: * All age =0 Chicks=1 Grower=2 Adult=3
**Season: 1=dry season 2=short rain 3= long rain season 4=every season

3. What is done with sick chickens (possible to select many)? _____a= isolate from healthy one b=eaten; c=sold ;d= treated; e =do nothing f=other _____
4. What is done with dead chickens?_____ a=buried b=bunt c= thrown in to garbage d= eaten to dog and cats e= thrown away in to ditch along road side e=other _____
5. If ND was not mentioned in Q2b
   i. Ask if the respondent knows or not ND? _____ (1=Yes; 0=No).
   ii. If yes, can you describe the clinical signs? _____
   (Green watery diarrhea, twisted neck, sudden mortality of several birds …)
6. If ND was mentioned in Q2 b
   i. when was ND last occur in your flock? ___a= <3mths; b=3-6mths ago; c= before 6mths
   ii. How did it affect your flock/ mortality rate? _____ (a=Killed out the whole flock; b=Killed more than half of the flock; c=killed less than half the flock; d=No mortality)
   iii. What was the suggested/suspected source of infection (could be many)? ___ (a= I don't know; b= incoming chicken from market (unsold/purchased); c= gift chicken; d= contact with neighboring household chicken; e= trader visit, f= Contamination by scavengers (dog &cat) g= exchanged cock h: Others(specify)_____
7. i. Do you have access to veterinary services for your chicken? _____(1= Yes; 0=No)
   ii. If no, what do you do when your chicken gets sick? _____ (1= nothing; 2= buy drugs from private pharmacy; 3=provide home made traditional medicine 4=other (specify)_____
8. i. Did you vaccinate your flock against any chicken disease in the last eight months? ____ (1=Yes ; 0=No)
   ii. If yes, could you mention the disease, type and frequency of vaccination?

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Disease vaccinated for	Vaccine *	Frequency**	Vaccinator***

NB: * administration form 1= drinking water, 2= intra-ocular, 3= injection, 4= spray

** 1=Sometimes, 2=once per year, 3=during an outbreak

*** 1= veterinarian, 2= technician/DA, 3= farmers, 4= other specify_____

Part IV. Others

1. Does your flock of chickens mix with your nearest neighbouring household keeping chickens? ____ (1=Yes; 0=No) If no, why?_____

   Estimate the average distance (meter) to the nearest neighbouring household keeping chickens. _____

2. Do you currently own other poultry species than chickens? ____ (1=Yes; 0=No)

3. a. Have you seen wild birds in the area where the village chickens are scavenging? ____ (1=Yes; 0=No)

   b. If yes, is there mixing between your chicken and wild birds? ____ (1=Yes ; 0=No)

4. a. Middlemen/trader visit to your village/flock since our last visit? ____ (1=Yes; 0=No)

   b. If yes, how often do they visit per month (in days) to your village? _____

4. Do you own dog currently? ____ (1=Yes; 0=No)
5. In the face of suspected outbreak of chicken disease in your village what do you do?_____ a= do nothing b=sell c=salvages slaughtering d= try preventive treatment e=confine at home f=other (specify) _____

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Appendix B. Estimation of evolutionary divergence between Ethiopian isolates and different genotypes (F gene 1047bp), Tamura-Nei model (Tamura *et al*., 1993).

Maximal Distance	ETH	ETH_303	ETH_762	I	II	II a	III	IV	IX	V	VI	VII	XI
ETH Isolates	0.054	0.128	0.161	0.194	0.200	0.205	0.193	0.158	0.175	0.179	0.148	0.163	0.220
ETH_303	0.128	0.000	0.125	0.165	0.181	0.174	0.153	0.126	0.143	0.143	0.061	0.118	0.195
ETH_762	0.161	0.125	0.000	0.169	0.189	0.183	0.153	0.125	0.141	0.156	0.138	0.116	0.199
I	0.194	0.165	0.169	0.063	0.140	0.120	0.115	0.096	0.108	0.193	0.182	0.173	0.180
II	0.200	0.181	0.189	0.140	0.033	0.120	0.146	0.134	0.127	0.197	0.193	0.191	0.201
II a	0.205	0.174	0.183	0.120	0.120	0.038	0.136	0.123	0.122	0.207	0.189	0.181	0.193
III	0.193	0.153	0.153	0.115	0.146	0.136	0.002	0.079	0.090	0.175	0.167	0.158	0.161
IV	0.158	0.126	0.125	0.096	0.134	0.123	0.079	0.016	0.076	0.155	0.141	0.134	0.113
IX	0.175	0.143	0.141	0.108	0.127	0.122	0.090	0.076	0.003	0.171	0.160	0.154	0.143
V	0.179	0.143	0.156	0.193	0.197	0.207	0.175	0.155	0.171	0.041	0.166	0.168	0.206
VI	0.148	0.061	0.138	0.182	0.193	0.189	0.167	0.141	0.160	0.166	0.052	0.134	0.207
VII	0.163	0.118	0.116	0.173	0.191	0.181	0.158	0.134	0.154	0.168	0.134	0.102	0.209
XI	0.220	0.195	0.199	0.180	0.201	0.193	0.161	0.113	0.143	0.206	0.207	0.209	0.027

Minimal Distance	ETH	ETH_303	ETH_762	I	II	II a	III	IV	IX	V	VI	VII	XI
ETH Isolates	0.000	0.118	0.148	0.166	0.183	0.185	0.181	0.141	0.158	0.158	0.128	0.133	0.195
ETH_303	0.118	0.000	0.125	0.158	0.169	0.170	0.153	0.122	0.142	0.125	0.050	0.111	0.191
ETH_762	0.148	0.125	0.000	0.159	0.177	0.175	0.153	0.125	0.138	0.137	0.125	0.041	0.196
I	0.166	0.158	0.159	0.000	0.113	0.104	0.108	0.091	0.095	0.163	0.158	0.140	0.164
II	0.183	0.169	0.177	0.113	0.000	0.103	0.138	0.123	0.118	0.176	0.167	0.170	0.184
II a	0.185	0.170	0.175	0.104	0.103	0.000	0.123	0.114	0.114	0.183	0.173	0.159	0.183
III	0.181	0.153	0.153	0.108	0.138	0.123	0.000	0.074	0.088	0.163	0.157	0.141	0.153
IV	0.141	0.122	0.125	0.091	0.123	0.114	0.074	0.000	0.072	0.139	0.122	0.113	0.104
IX	0.158	0.142	0.138	0.095	0.118	0.114	0.088	0.072	0.000	0.152	0.144	0.130	0.139
V	0.158	0.125	0.137	0.163	0.176	0.183	0.163	0.139	0.152	0.000	0.136	0.127	0.185
VI	0.128	0.050	0.125	0.158	0.167	0.173	0.157	0.122	0.144	0.136	0.000	0.106	0.184
VII	0.133	0.111	0.041	0.140	0.170	0.159	0.141	0.113	0.130	0.127	0.106	0.000	0.182
XI	0.195	0.191	0.196	0.164	0.184	0.183	0.153	0.104	0.139	0.185	0.184	0.182	0.000

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Appendix C. Model scripts for the Bayesian analysis of the two ELISA's and HI tests

a. Two population models with no dependence between tests

```
model {

y1[1:Q, 1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q, 1:Q], n1)
y2[1:Q, 1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q, 1:Q], n2)
y3[1:Q, 1:Q] ~ dmulti(p3[1:Q, 1:Q], n3)
y4[1:Q, 1:Q] ~ dmulti(p4[1:Q, 1:Q], n4)

p1[1,1,1] <- Prev1*(SeT1*SeT2)*SeT3 + (1-Prev1)*((1-SpT1)*(1-SpT2))*(1-SpT3)
p1[1,1,2] <- Prev1*(SeT1*SeT2)*(1-SeT3) + (1-Prev1)*((1-SpT1)*(1-SpT2))*(SpT3)
p1[1,2,1] <- Prev1*(SeT1*(1-SeT2))*SeT3 + (1-Prev1)*((1-SpT1)*SpT2)*(1-SpT3)
p1[1,2,2] <- Prev1*(SeT1*(1-SeT2))*(1-SeT3) + (1-Prev1)*((1-SpT1)*SpT2)*(SpT3)
p1[2,1,1] <- Prev1*((1-SeT1)*SeT2)*SeT3 + (1-Prev1)*(SpT1*(1-SpT2))*(1-SpT3)
p1[2,1,2] <- Prev1*((1-SeT1)*SeT2)*(1-SeT3) + (1-Prev1)*(SpT1*(1-SpT2))*SpT3
p1[2,2,1] <- Prev1*((1-SeT1)*(1-SeT2))*SeT3 + (1-Prev1)*(SpT1*SpT2)*(1-SpT3)
p1[2,2,2] <- 1-p1[1,1,1]-p1[1,1,2]-p1[1,2,1]-p1[1,2,2]-p1[2,1,1]-p1[2,1,2]-p1[2,2,1]

p2[1,1,1] <- Prev2*(SeT1*SeT2)*SeT3 + (1-Prev2)*((1-SpT1)*(1-SpT2))*(1-SpT3)
p2[1,1,2] <- Prev2*(SeT1*SeT2)*(1-SeT3) + (1-Prev2)*((1-SpT1)*(1-SpT2))*(SpT3)
p2[1,2,1] <- Prev2*(SeT1*(1-SeT2))*SeT3 + (1-Prev2)*((1-SpT1)*SpT2)*(1-SpT3)
p2[1,2,2] <- Prev2*(SeT1*(1-SeT2))*(1-SeT3) + (1-Prev2)*((1-SpT1)*SpT2)*(SpT3)
p2[2,1,1] <- Prev2*((1-SeT1)*SeT2)*SeT3 + (1-Prev2)*(SpT1*(1-SpT2))*(1-SpT3)
p2[2,1,2] <- Prev2*((1-SeT1)*SeT2)*(1-SeT3) + (1-Prev2)*(SpT1*(1-SpT2))*SpT3
p2[2,2,1] <- Prev2*((1-SeT1)*(1-SeT2))*SeT3 + (1-Prev2)*(SpT1*SpT2)*(1-SpT3)
p2[2,2,2] <- 1-p2[1,1,1]-p2[1,1,2]-p2[1,2,1]-p2[1,2,2]-p2[2,1,1]-p2[2,1,2]-p2[2,2,1]

p3[1,1] <- Prev1*SeT1*SeT2 + (1-Prev1)*(1-SpT1)*(1-SpT2)
p3[1,2] <- Prev1*SeT1*(1-SeT2) + (1-Prev1)*(1-SpT1)*SpT2
p3[2,1] <- Prev1*(1-SeT1)*SeT2 + (1-Prev1)*SpT1*(1-SpT2)
p3[2,2] <- 1-p3[1,1]-p3[1,2]-p3[2,1]

p4[1,1] <- Prev2*SeT1*SeT2 + (1-Prev2)*(1-SpT1)*(1-SpT2)
p4[1,2] <- Prev2*SeT1*(1-SeT2) + (1-Prev2)*(1-SpT1)*SpT2
p4[2,1] <- Prev2*(1-SeT1)*SeT2 + (1-Prev2)*SpT1*(1-SpT2)
p4[2,2] <- 1-p4[1,1]-p4[1,2]-p4[2,1]

SeT1~dbeta(23.35,1.46) # B_Elisa mode = 0.98, 95% sure > 0.85
SeT2~dbeta(23.9, 5.04) # HI mode = 0.85, 95% sure > 0.70
SeT3~dbeta(36.7,2.88) # I_Elisa mode = 0.95, 95% sure > 0.85

SpT1~dbeta(34.17, 1.36) # B_Elisa mode = 0.99, 95% sure > 0.90
SpT2~dbeta(34.17, 1.36) # HI mode = 0.99, 95% sure > 0.90
SpT3~dbeta(14.52,1.37) # I_Elisa mode = 0.99, 95% sure > 0.80

Prev1~dbeta(1.49,5.38) #wet season mode = 0.10, 95% sure < 0.50
Prev2~dbeta(1.93,6.25) #dry season mode = 0.15, 95% sure < 0.50

}
# data
# T1 = belisa; T2 = HI; T3 = ielisa
# n1 = sais 1; n2 = sais 2

list(n1=233, n2=204, n3=1018, n4=881, Q=2,
y1=structure(.Data=c(7,0,6,0,12,2,167,10),.Dim=c(2,2,2)),
y2=structure(.Data=c(7,0,7,0,26,2,181,10),.Dim=c(2,2,2)),
y3=structure(.Data=c(41,15,37,925),.Dim=c(2,2)),
y4=structure(.Data=c(73,23,10,775),.Dim=c(2,2))
)


# initials 1
list(SeT1=0.5, SpT1=0.5, SeT2=0.90, SpT2=0.95, SeT3=0.5, SpT3=0.5, Prev1=0.1, Prev2=0.2)

# initials 2 (alternative)
list(SeT1=0.8, SpT1=0.8, SeT2=0.5, SpT2=0.5, SeT3=0.8, SpT3=0.9, Prev1=0.3, Prev2=0.1)
```


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b. Four populations models with no dependence between tests

```
model {

y1[1:Q, 1:Q, 1:Q] ~ dmulti(p1[1:Q, 1:Q, 1:Q], n1)
y2[1:Q, 1:Q, 1:Q] ~ dmulti(p2[1:Q, 1:Q, 1:Q], n2)
y3[1:Q, 1:Q] ~ dmulti(p3[1:Q, 1:Q], n3)
y4[1:Q, 1:Q] ~ dmulti(p4[1:Q, 1:Q], n4)

p1[1,1,1] <- Prev1*SeT1*SeT2*SeT3 + (1-Prev1)*((1-SpT1)*(1-SpT2))*(1-SpT3)
p1[1,1,2] <- Prev1*SeT1*SeT2*(1-SeT3) + (1-Prev1)*(1-SpT1)*(1-SpT2)*SpT3
p1[1,2,1] <- Prev1*SeT1*(1-SeT2)*SeT3 + (1-Prev1)*(1-SpT1)*SpT2*(1-SpT3)
p1[1,2,2] <- Prev1*SeT1*(1-SeT2)*(1-SeT3) + (1-Prev1)*(1-SpT1)*SpT2*SpT3
p1[2,1,1] <- Prev1*(1-SeT1)*SeT2*SeT3 + (1-Prev1)*SpT1*(1-SpT2)*(1-SpT3)
p1[2,1,2] <- Prev1*(1-SeT1)*SeT2*(1-SeT3) + (1-Prev1)*SpT1*(1-SpT2)*SpT3
p1[2,2,1] <- Prev1*(1-SeT1)*(1-SeT2)*SeT3 + (1-Prev1)*SpT1*SpT2*(1-SpT3)
p1[2,2,2] <- 1-p1[1,1,1]-p1[1,1,2]-p1[1,2,1]-p1[1,2,2]-p1[2,1,1]-p1[2,1,2]-p1[2,2,1]

p2[1,1,1] <- Prev2*SeT1*SeT2*SeT3 + (1-Prev2)*(1-SpT1)*(1-SpT2)*(1-SpT3)
p2[1,1,2] <- Prev2*SeT1*SeT2*(1-SeT3) + (1-Prev2)*(1-SpT1)*(1-SpT2)*(SpT3)
p2[1,2,1] <- Prev2*SeT1*(1-SeT2)*SeT3 + (1-Prev2)*(1-SpT1)*SpT2*(1-SpT3)
p2[1,2,2] <- Prev2*SeT1*(1-SeT2)*(1-SeT3) + (1-Prev2)*(1-SpT1)*SpT2*(SpT3)
p2[2,1,1] <- Prev2*(1-SeT1)*SeT2*SeT3 + (1-Prev2)*SpT1*(1-SpT2)*(1-SpT3)
p2[2,1,2] <- Prev2*(1-SeT1)*SeT2*(1-SeT3) + (1-Prev2)*SpT1*(1-SpT2)*SpT3
p2[2,2,1] <- Prev2*(1-SeT1)*(1-SeT2)*SeT3 + (1-Prev2)*SpT1*SpT2*(1-SpT3)
p2[2,2,2] <- 1-p2[1,1,1]-p2[1,1,2]-p2[1,2,1]-p2[1,2,2]-p2[2,1,1]-p2[2,1,2]-p2[2,2,1]

p3[1,1] <- Prev3*SeT1*SeT2 + (1-Prev3)*(1-SpT1)*(1-SpT2)
p3[1,2] <- Prev3*SeT1*(1-SeT2) + (1-Prev3)*(1-SpT1)*SpT2
p3[2,1] <- Prev3*(1-SeT1)*SeT2 + (1-Prev3)*SpT1*(1-SpT2)
p3[2,2] <- 1-p3[1,1]-p3[1,2]-p3[2,1]

p4[1,1] <- Prev4*SeT1*SeT2 + (1-Prev4)*(1-SpT1)*(1-SpT2)
p4[1,2] <- Prev4*SeT1*(1-SeT2) + (1-Prev4)*(1-SpT1)*SpT2
p4[2,1] <- Prev4*(1-SeT1)*SeT2 + (1-Prev4)*SpT1*(1-SpT2)
p4[2,2] <- 1-p4[1,1]-p4[1,2]-p4[2,1]

SeT1~dbeta(23.35,1.46) # B_Elisa mode = 0.98, 95% sure > 0.85
SeT2~dbeta(23.9, 5.04) # HI mode = 0.85, 95% sure > 0.70
SeT3~dbeta(36.7,2.88) # I_Elisa mode = 0.95, 95% sure > 0.85

SpT1~dbeta(34.17, 1.36) # B_Elisa mode = 0.99, 95% sure > 0.90
SpT2~dbeta(34.17, 1.36) # HI mode = 0.99, 95% sure > 0.90
SpT3~dbeta(14.52,1.14) # I_Elisa mode = 0.99, 95% sure > 0.80

Prev1~dbeta(1.49,5.38) #wet season mode = 0.10, 95% sure < 0.50
Prev2~dbeta(1.93,6.25) #dry season mode = 0.15, 95% sure < 0.50
Prev3~dbeta(1.49,5.38) #wet season mode = 0.10, 95% sure < 0.50
Prev4~dbeta(1.93,6.25) #dry season mode = 0.15, 95% sure < 0.50
}


# data
# T1 = belisa; T2 = HI; T3 = ielisa
# n1 = sais 1; n2 = sais 2

list(n1=204, n2=233, n3=1018, n4=881, Q=2,
y1=structure(.Data=c(7,0,6,0,12,2,167,10),.Dim=c(2,2,2)),
y2=structure(.Data=c(7,0,7,0,26,2,181,10),.Dim=c(2,2,2)),
y3=structure(.Data=c(41,15,37,925),.Dim=c(2,2)),
y4=structure(.Data=c(73,23,10,775),.Dim=c(2,2))
)

# initials 1
list(SeT1=0.5, SpT1=0.5, SeT2=0.90, SpT2=0.95, SeT3=0.5, SpT3=0.5, Prev1=0.1, Prev2=0.2, Prev3=0.15, Prev4=0.25)

# initials 2 (alternative)
list(SeT1=0.8, SpT1=0.8, SeT2=0.5, SpT2=0.5, SeT3=0.8, SpT3=0.9, Prev1=0.3, Prev2=0.1, Prev3=0.40, Prev4=0.05)
```


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Appendix D. Live poultry market questionnaire to traders for contact network study

Woreda	Residence Town/Kebelle/PA		Date of Interview (dd/mm/yy)
Interviewee: Name Sex	 ☐Male ☐Female		
Market place/Name		Code	ETM:_____
Interviewer name			

A. General questions

6. a. Has the trader been interviewed in another market place in the past week?☐

☐Yes ☐No

b. If yes, which market place? _____

7. Trader activity

a. Product(s) traded: ☐eggs only ☐chicken only ☐both

b. For the trader live chicken trading is considered as _____ activity

☐Regular ☐Irregular ☐Occasional (eg during holidays etc)

8. a. For regular chicken traders in which months of the year (possible to select months) do you buy more/less chicken and why?_____

_____

Action	Oct	Nov	Dec	Jan	Feb	Mar	Apr	May	June	Jul	Agu	Sep
more	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐
less	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐	☐

b. From trader's observation ask what affects chicken supply to market mostly? ☐ Holidays, ☐farming season, ☐disease occurrence, ☐other specify_____

c. How do you rate the supply of chicken to the market today compared to other times of the year?

☐☐Lowest ☐ Low ☐ comparable to other time of the year ☐higher

If the supply is considered as lowest or higher why?

_____

B. **Specific questions: Poultry trade characteristics**

9. a. Purpose of traders visit to this market(where interview is being done): ____ ☐Buy ☐Sell ☐Both

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b. Which markets (including **market being visited**) did you visit for buying or selling of chickens (**in the last one week)**?

#	Purchase/buy at			Sell or Sold at			Remark
	Market/ Place*	Market day(s)	Quantity purchased	Market/ place	Market day(s)	Quantity	

N.B: * include market being visited for interview in the list as required

c. Main buyers for your products (tick boxes and rank according to amount sold to each) at sell market(s), during these period of the year.

	Town consumers	Restaurant/pastry	super markets/ shops	another trader	Individual breeder	Others (Specify)
	□	□	□	□	□	□
Rank*						

N.B: * 1 is first rank

C. Biosecurity issues

10. How do you transport poultry products (live chicken and eggs) from purchased market (tick the box)? On/by:

□On foot □By bike □motor □car □animal □Other (specify)_____

11. a. Do you keep chickens (collected from different markets) as stock at home until sell ?___□□Yes □No

b. If yes, how long is that on average (in days)? _____

c. Do you own chicken at home? ____□□Yes □□No

d. If yes, do you allow them to run together with those for trade? _____ □Yes □□No

12. Additional comments, suggestion and/or observations

_____
_____
_____
_____
_____
_____

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Appendix E. The geocodes and location details of all the markets included in the contact network study

Id_market	Woreda	Market names	Lat_dd (N)	Long_dd(E)
ETM-01	Akaki	Akaki	8.898611	38.78337
ETM-02	Akaki	Dukem	8.8	38.9
ETM-03	Adaa	Debre Zeit	8.733591	38.98346
ETM-04	Adaa	Ardaga	8.816671	39.16673
ETM-05	Adaa	Dire	8.683444	38.8835
ETM-06	Adaa	Bakajo	8.600254	38.93347
ETM-07	Adaa	Godino	8.850077	39.03342
ETM-08	Adaa	Hidi	8.783492	39.08336
ETM-09	Adaa	Bole/Tuludimitu	8.883333	39.15
ETM-10	Gimbichu	Chefedonsa	9.600224	39.20012
ETM-11	Liben	Ziquela	8.550256	38.08349
ETM-12	Liben	Adulala	8.533402	38.90003
ETM-13	Lome/Ejere	Ejere	8.783333	39.25013
ETM-14	Lome	Koka	8.433454	39.01692
ETM-15	Lome	Mojo	8.583584	39.11682
ETM-16	Bora	Alemtena	8.300021	38.93357
ETM-17	Dugda	Abura	8.033577	39.00018
ETM-18	Dugda	Meki	8.133574	38.8002
ETM-19	ATJC	Ziway	7.933375	38.70023
ETM-20	ATJC	Adami Tulu	7.850175	38.70009
ETM-21	ATJC	Bulbula	7.716726	38.65006
ETM-22	ATJC	Abosa	8.016745	38.71681
ETM-23	ATJC	Hoitu	8.183543	39.26672
ETM-24	ATJC	Jido	7.700181	38.46673
ETM-25	Mareko	Qoshe	8.00014	38.5169
ETM-26	Lanfuru	Tora	7.850118	38.4167
ETM-27	Meskan	Enseno	7.616876	38.46674
ETM-28	SW-Arsi	Arsi Negele	7.350156	38.6668
ETM-29	Shashemene	Shasmene	7.200058	38.58355
ETM-30	Adama	Nazreth	8.550046	39.26671
ETM-31	Boset	Doni	8.500111	39.55004
ETM-32	Boset	Wolanchiti	8.466675	39.45004
ETM-33	Boset	Bofa	8.316881	39.31672
ETM-34	Dodota	Dhera	8.316881	39.31672
ETM-35	Diksis	Diksis	8.05	39.58333
ETM-36	Bale	Arsi Bale	8.25	39.58333
ETM-37	Seru	Seru	7.666667	40.2
ETM-39	Sibu Sire	Sire	8.316667	39.48333
ETM-40	Itaya	Itaya	8.116914	39.21694
ETM-41	Qore	Qore	7.35026	39.53357
ETM-42	Kofole	Kofole	7.116811	39.30006
ETM-43	Kuyera	Kuyera	7.266686	38.65011
ETM-44	Shala	Aje	7.283547	38.35017
ETM-45	Wolyta	Sodo	6.9	37.75
ETM-46	Shire	Shire	7.400011	39.63358
ETM-47	Siraru	Rophe	7.216943	38.18361
ETM-48	Akaki	Aba Samuel	8.75	38.71667
ETM-50	Minjar	Balchi	8.916667	39.36667
ETM-51	Yaya gulele	Chancho	9.566667	38.68333
ETM-52	mukaturi	Dubar	9.483333	38.08333
ETM-53	Dodota	Awash melkasa	8.666788	39.55016
ETM-55	Akaki	Abusera	8.650061	38.76669
ETM-56	Asela	Asela	7.950036	39.13345
ETM-57	Butajira	Butajira	8.116667	38.36673
ETM-99	AA	Addis Ababa	9.016782	38.78351

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Appendix F. Live bird markets in the contact networks evaluated and ranked according to the their centrality measures and trade characteristics

a) Markets evaluated based on centrality measures and out-going parameters during period one

Nodes	Market names	Out Degree	Nout Degree[a]	NW-1 Fsend[b]	NW-1 Qsend[c]	Clus Coef	Betweenness	nBetweenness[d]
ETM-18	Meki	6	11.11	33	3427	0.118	33.167	1.16
ETM-25	Qoshe	6	11.11	16	434	0.200	0.000	0.00
ETM-16	AlemTena	5	9.26	21	1752	0.300	0.000	0.00
ETM-31	Doni	5	9.26	13	424	0.200	0.000	0.00
ETM-17	Abura	5	9.26	7	174	0.150	0.000	0.00
ETM-28	Arsinegele	4	7.41	6	240	0.100	21.833	0.76
ETM-14	Koka	4	7.41	13	405	0.167	0.000	0.00
ETM-06	Bekajo	4	7.41	13	315	0.167	0.000	0.00
ETM-26	Tora	4	7.41	10	301	0.000	0.000	0.00
ETM-12	Adulala	4	7.41	9	504	0.250	0.000	0.00
ETM-21	Bulbula	3	5.56	15	564	0.200	8.500	0.30
ETM-05	Dire	3	5.56	24	580	0.233	3.333	0.12
ETM-08	Hidi	3	5.56	8	146	0.083	2.000	0.07
ETM-34	Dehra	3	5.56	16	438	0.333	0.000	0.00
ETM-20	Adami Tulu	3	5.56	10	167	0.667	0.000	0.00
ETM-11	Ziquala	3	5.56	9	192	0.500	0.000	0.00
ETM-23	Hoitu	3	5.56	6	170	0.167	0.000	0.00
ETM-24	Jido	3	5.56	4	297	0.167	0.000	0.00
ETM-45	Sodo	3	5.56	3	490	0.167	0.000	0.00
ETM-10	Chefe donsa	3	5.56	3	355	0.167	0.000	0.00
ETM-29	Shashemene	2	3.70	2	162	0.027	26.000	0.91
ETM-19	Ziway	2	3.70	6	269	0.167	14.500	0.51
ETM-15	Mojo	2	3.70	11	788	0.133	2.333	0.08
ETM-43	Kuyera	2	3.70	6	116	1.000	0.000	0.00
ETM-35	Diksis	2	3.70	5	490	0.000	0.000	0.00
ETM-04	Ardaga	2	3.70	5	36	0.500	0.000	0.00
ETM-13	Ejere	2	3.70	4	102	0.000	0.000	0.00
ETM-39	Sire	2	3.70	2	97	0.500	0.000	0.00
ETM-03	Debre Zeit	1	1.85	1	80	0.052	5.333	0.19
ETM-27	Enseno	1	1.85	2	330	0.167	0.500	0.02
ETM-02	Dukem	1	1.85	1	50	0.150	0.500	0.00
ETM-41	Qore	1	1.85	10	472	0.000	0.000	0.00
ETM-42	Kofole	1	1.85	10	359	0.000	0.000	0.00
ETM-32	Wolanchiti	1	1.85	10	259	0.000	0.000	0.00
ETM-07	Godino	1	1.85	7	150	0.000	0.000	0.00
ETM-22	Abosa	1	1.85	7	30	0.000	0.000	0.00
ETM-09	Bole	1	1.85	6	178	0.000	0.000	0.00
ETM-33	Bofa	1	1.85	4	133	0.000	0.000	0.00
ETM-37	Seru	1	1.85	3	108	0.000	0.000	0.00
ETM-49	Shenkora	1	1.85	3	70	0.000	0.000	0.00
ETM-44	Aje	1	1.85	3	68	0.000	0.000	0.00
ETM-36	Arsi Bale	1	1.85	2	177	0.000	0.000	0.00
ETM-40	Itaya	1	1.85	2	70	0.000	0.000	0.00
ETM-46	Shire	1	1.85	1	70	0.000	0.000	0.00
ETM-48	Aba Samuel	1	1.85	1	48	0.000	0.000	0.00
ETM-52	Dubar	1	1.85	1	30	0.000	0.000	0.00
ETM-51	Chancho	1	1.85	1	20	0.000	0.000	0.00
ETM-55	Abusera	1	1.85	1	20	0.000	0.000	0.00
ETM-50	Balchi	1	1.85	1	15	0.000	0.000	0.00
ETM-53	Awash Melka	1	1.85	1	10	0.000	0.000	0.00
ETM-99	Addis Ababa	0	0.00	0	0	0.059	0.000	0.00
ETM-01	Akaki	0	0.00	0	0	0.034	0.000	0.00
ETM-30	Amede	0	0.00	0	0	0.018	0.000	0.00
ETM-57	Butajira	0	0.00	0	0	0.000	0.000	0.00
ETM-56	Asela	0	0.00	0	0		0.000	0.00

[a] normalized out-degree(percentage)
b off-market trader movement
[c] off-market quantity of chickens moved
[d] normalized betweenness

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b) Markets evaluated based on centrality measures and in-coming parameters during period one

Nodes	Market names	In Degree	Nin Degree[a]	NW-1 Freceive[b]	NW-1 Qreceive[c]	Clus Coef	Betweenness	nBetweenness[d]
ETM-01	Akaki	24	44.44	57	4436	0.034	0.00	0.00
ETM-99	AddiAdaba	18	33.33	64	5106	0.059	0.00	0.00
ETM-03	Debrezeit	17	31.48	59	1805	0.052	5.33	0.19
ETM-30	Nazreth	11	20.37	47	1495	0.018	0.00	0.00
ETM-29	Shashemene	10	18.52	33	1252	0.027	26.00	0.91
ETM-18	Meki	6	11.11	11	344	0.118	33.17	1.16
ETM-19	Ziway	6	11.11	31	669	0.167	14.50	0.51
ETM-15	Mojo	4	7.41	8	219	0.133	2.33	0.08
ETM-02	Dukem	4	7.41	8	155	0.150	0.50	0.02
ETM-28	Arsinegele	3	5.56	11	272	0.100	21.83	0.76
ETM-05	Dire	3	5.56	10	153	0.233	3.33	0.12
ETM-21	Bulbula	2	3.70	4	73	0.200	8.50	0.30
ETM-27	Enseno	2	3.70	6	51	0.167	0.50	0.02
ETM-57	Butajira	2	3.70	3	76	0.000	0.00	0.00
ETM-08	Hidi	1	1.85	2	6	0.083	2.00	0.07
ETM-56	Asela	1	1.85	3	60		0.00	0.00
ETM-34	Dhera	1	1.85	1	10	0.333	0.00	0.00
ETM-43	Kuyera	0	0.00	0	0	1.000	0.00	0.00
ETM-20	Adami Tulu	0	0.00	0	0	0.667	0.00	0.00
ETM-04	Ardaga	0	0.00	0	0	0.500	0.00	0.00
ETM-11	Ziquala	0	0.00	0	0	0.500	0.00	0.00
ETM-39	Sire	0	0.00	0	0	0.500	0.00	0.00
ETM-16	Alemtena	0	0.00	0	0	0.300	0.00	0.00
ETM-12	Aduala	0	0.00	0	0	0.250	0.00	0.00
ETM-25	Qoshe	0	0.00	0	0	0.200	0.00	0.00
ETM-31	Doni	0	0.00	0	0	0.200	0.00	0.00
ETM-06	Bakajo	0	0.00	0	0	0.167	0.00	0.00
ETM-10	Chefedonsa	0	0.00	0	0	0.167	0.00	0.00
ETM-14	Koka	0	0.00	0	0	0.167	0.00	0.00
ETM-23	Hoitu	0	0.00	0	0	0.167	0.00	0.00
ETM-24	Jodo	0	0.00	0	0	0.167	0.00	0.00
ETM-45	Sodo	0	0.00	0	0	0.167	0.00	0.00
ETM-17	Abura	0	0.00	0	0	0.150	0.00	0.00
ETM-13	Ejere	0	0.00	0	0	0.000	0.00	0.00
ETM-26	Tora	0	0.00	0	0	0.000	0.00	0.00
ETM-35	Diksis	0	0.00	0	0	0.000	0.00	0.00
ETM-07	Godino	0	0.00	0	0	0.000	0.00	0.00
ETM-09	Bole	0	0.00	0	0	0.000	0.00	0.00
ETM-22	Abosa	0	0.00	0	0	0.000	0.00	0.00
ETM-32	Wolanchiti	0	0.00	0	0	0.000	0.00	0.00
ETM-33	Bofa	0	0.00	0	0	0.000	0.00	0.00
ETM-36	Arsi Bala	0	0.00	0	0	0.000	0.00	0.00
ETM-37	Seru	0	0.00	0	0	0.000	0.00	0.00
ETM-40	Itaya	0	0.00	0	0	0.000	0.00	0.00
ETM-41	Qore	0	0.00	0	0	0.000	0.00	0.00
ETM-42	Kofele	0	0.00	0	0	0.000	0.00	0.00
ETM-44	Aje	0	0.00	0	0	0.000	0.00	0.00
ETM-46	Shire	0	0.00	0	0	0.000	0.00	0.00
ETM-48	Aba Samuel	0	0.00	0	0	0.000	0.00	0.00
ETM-49	Shenkora	0	0.00	0	0	0.000	0.00	0.00
ETM-50	Balchi	0	0.00	0	0	0.000	0.00	0.00
ETM-51	Chancho	0	0.00	0	0	0.000	0.00	0.00
ETM-52	Dubar	0	0.00	0	0	0.000	0.00	0.00
ETM-53	Awash melaka	0	0.00	0	0	0.000	0.00	0.00
ETM-55	Abusera	0	0.00	0	0	0.000	0.00	0.00

[a] normalized in-degree (percentage) b on-market trader movement

[c] on-market quantity of chickens moved [d] normalized betweenness

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c) Markets evaluated based on centrality measures and out-going parameters during period two

Nodes	Market names	Out Degree	Nout Degree[a]	NW-2 Fsend[b]	NW-2 Qsend[c]	Clus Coef	Betweenness	nBetweenness[d]
ETM-21	Bulbula	7	16.67	13	494	0.143	11.00	0.64
ETM-18	Meki	6	14.29	13	377	0.144	44.83	2.60
ETM-25	Qoshe	6	14.29	12	317	0.233	0.00	0.00
ETM-16	AlemTena	5	11.90	6	125	0.250	11.00	0.64
ETM-20	Adamitulu	4	9.52	8	351	0.350	0.83	0.05
ETM-26	Tora	4	9.52	14	795	0.350	0.00	0.00
ETM-22	Abosa	4	9.52	7	73	0.333	0.00	0.00
ETM-28	Arsinegele	3	7.14	6	255	0.100	16.00	0.93
ETM-17	Abura	3	7.14	6	238	0.167	0.00	0.00
ETM-09	Tuludimitu	3	7.14	4	163	0.000	0.00	1.30
ETM-19	Ziway	2	4.76	5	110	0.167	22.33	0.00
ETM-31	Doni	2	4.76	12	188	0.500	0.00	0.00
ETM-33	Bofa	2	4.76	8	104	0.500	0.00	0.00
ETM-34	Dhera	2	4.76	7	119	0.000	0.00	1.30
ETM-05	Dire	2	4.76	7	73	0.000	0.00	0.00
ETM-14	Koka	2	4.76	7	60	0.333	0.00	0.00
ETM-27	Enseno	2	4.76	4	172	0.417	0.00	0.00
ETM-42	Kofole	2	4.76	4	135	1.000	0.00	0.00
ETM-35	Diksis	2	4.76	2	90	0.000	0.00	0.00
ETM-12	Adulala	2	4.76	2	58	0.000	0.00	0.00
ETM-29	Shashmene	1	2.38	1	10	0.048	12.00	0.70
ETM-32	Wolanchiti	1	2.38	7	65	0.000	0.00	0.00
ETM-23	Hoitu	1	2.38	4	62	0.000	0.00	0.00
ETM-41	Qore	1	2.38	3	120	0.000	0.00	0.00
ETM-47	Rophe	1	2.38	3	62	0.000	0.00	0.00
ETM-44	Aje	1	2.38	3	42	0.000	0.00	0.00
ETM-40	Itaya	1	2.38	2	40	0.000	0.00	0.00
ETM-45	Sodo	1	2.38	1	123	0.000	0.00	0.00
ETM-24	Jido	1	2.38	1	70	0.000	0.00	0.00
ETM-36	Arsi Bala	1	2.38	1	50	0.000	0.00	0.00
ETM-37	Seru	1	2.38	1	45	0.000	0.00	0.00
ETM-43	Kuyera	1	2.38	1	25	0.000	0.00	0.00
ETM-10	Chefe Donsa	1	2.38	1	22	0.000	0.00	0.00
ETM-02	Dukem	1	2.38	1	15	0.000	0.00	0.00
ETM-06	Bakajo	1	2.38	1	15	0.000	0.00	0.00
ETM-11	Ziquala	1	2.38	1	15	0.000	0.00	0.00
ETM-39	Sire	1	2.38	1	10	0.000	0.00	0.00
ETM-15	Mojo	0	0.00	0	0	0.500	0.00	0.00
ETM-99	Addis Ababa	0	0.00	0	0	0.122	0.00	0.00
ETM-01	Akaki	0	0.00	0	0	0.053	0.00	0.00
ETM-03	Debre Zeit	0	0.00	0	0	0.042	0.00	0.00
ETM-30	Amede	0	0.00	0	0	0.013	0.00	0.00
ETM-56	Asela	0	0.00	0	0		0.00	0.00

[a] normalized in-degree (percentage) b on-market trader movement
[c] on-market quantity of chickens moved [d] normalized betweenness

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d) Markets evaluated based on centrality measures and in-coming parameters during period one

Nodes	Market names	In Degree	Nin Degree[a]	NW-2 Freceive[b]	NW-2 Qreceive[c]	Clus Coef	Betweenness	nBetweenness[d]
ETM-30	Nazreth	13	30.95	41	784	0.013	0.00	0.00
ETM-01	Akaki	12	28.57	21	859	0.053	0.00	0.00
ETM-99	Addis Ababa	10	23.81	16	979	0.122	0.00	0.00
ETM-03	Debrezeit	9	21.43	16	406	0.042	0.00	0.00
ETM-19	Ziway	8	19.05	23	711	0.167	22.33	1.30
ETM-29	Shashmene	7	16.67	17	577	0.048	12.00	0.70
ETM-18	Meki	6	14.29	13	272	0.144	44.83	2.60
ETM-28	Arsinegele	4	9.52	4	85	0.100	16.00	0.93
ETM-15	Mojo	3	7.14	9	75	0.500	0.00	0.00
ETM-27	Enseno	2	4.76	6	110	0.417	0.00	0.00
ETM-21	Bulbula	1	2.38	4	62	0.143	11.00	0.64
ETM-16	Alemtena	1	2.38	3	87	0.250	11.00	0.64
ETM-20	Adamitulu	1	2.38	1	2	0.350	0.83	0.05
ETM-33	Bofa	1	2.38	2	19	0.500	0.00	0.00
ETM-56	Asela	1	2.38	1	25		0.00	0.00
ETM-26	Tora	1	2.38	1	20	0.350	0.00	0.00
ETM-14	Koka	1	2.38	1	10	0.333	0.00	0.00
ETM-31	Doni	1	2.38	1	5	0.500	0.00	0.00
ETM-42	Kofele	0	0.00	0	0	1.000	0.00	0.00
ETM-22	Abosa	0	0.00	0	0	0.333	0.00	0.00
ETM-25	Qoshe	0	0.00	0	0	0.233	0.00	0.00
ETM-17	Abura	0	0.00	0	0	0.167	0.00	0.00
ETM-05	Dire	0	0.00	0	0	0.000	0.00	0.00
ETM-09	Bole	0	0.00	0	0	0.000	0.00	0.00
ETM-12	Adulala	0	0.00	0	0	0.000	0.00	0.00
ETM-34	Dhera	0	0.00	0	0	0.000	0.00	0.00
ETM-35	Diksisi	0	0.00	0	0	0.000	0.00	0.00
ETM-02	Dukem	0	0.00	0	0	0.000	0.00	0.00
ETM-06	Bakajo	0	0.00	0	0	0.000	0.00	0.00
ETM-10	Chefedonsa	0	0.00	0	0	0.000	0.00	0.00
ETM-11	Ziquala	0	0.00	0	0	0.000	0.00	0.00
ETM-23	Hoitu	0	0.00	0	0	0.000	0.00	0.00
ETM-24	Jodo	0	0.00	0	0	0.000	0.00	0.00
ETM-32	Wolanchiti	0	0.00	0	0	0.000	0.00	0.00
ETM-36	Arsi Bala	0	0.00	0	0	0.000	0.00	0.00
ETM-37	Seru	0	0.00	0	0	0.000	0.00	0.00
ETM-39	Sire	0	0.00	0	0	0.000	0.00	0.00
ETM-40	Itaya	0	0.00	0	0	0.000	0.00	0.00
ETM-41	Qore	0	0.00	0	0	0.000	0.00	0.00
ETM-43	Kuyera	0	0.00	0	0	0.000	0.00	0.00
ETM-44	Aje	0	0.00	0	0	0.000	0.00	0.00
ETM-45	Sodo	0	0.00	0	0	0.000	0.00	0.00
ETM-47	Rophe	0	0.00	0	0		0.00	0.00

[a] normalized in-degree (percentage) b on-market trader movement
[c] on-market quantity of chickens moved [d] normalized betweenness

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Appendix G. Publications

Journals

Chaka' H., Goutard, F., Roger, F., Bisschop, S.P.R. and Thompson, P.N. 2012. Household-level risk factors for Newcastle disease virus seropositivity and incidence of Newcastle disease virus in backyard chicken flocks in Eastern Shewa Zone, Ethiopia. Preventive Veterinary Medicine. DOI:10.1016/j.prevetmed.2012.10.003.

Chaka' H., Goutard, F, Gil, P., Abolnik, C., Servan de Almeida, R., Bisschop, S.P.R. and Thompson, P.N. 2012. Serological and molecular investigation of Newcastle disease in household chicken flocks and associated markets in Eastern Shewa zone, Ethiopia. Tropical Animal Health and Production. DOI: 10.1007/s11250-012-0278-y.

Chaka, **H.,** Goutard, F., Bisschop, S.P.R. and Thompson, P.N. 2012. Seroprevalence of Newcastle disease and other infectious diseases in backyard chickens at markets in Eastern Shewa Zone, Ethiopia. Poultry Science, 91: 862-869.

Conference proceedings and posters

Chaka, H., Goutard, F., Roger, F., Bisschop, S.P.R. and Thompson, P. N. 2012. Household-level risk factors for Newcastle disease virus seropositivity and incidence in backyard chicken flocks in Eastern Shewa Zone, Ethiopia. 13th International Symposium on Veterinary Epidemiology and Economics (ISVEE), Maastricht, the Netherlands, 20-24 August 2012.

Chaka, H., Goutard, F. Bisschop, S.P.R. and Thompson, P.N. 2011. Serological and virological studies of Newcastle disease in backyard chickens, Ethiopia. 30th World Veterinary Congress, Cape Town, South Africa, 10-14 October, 2011.

Chaka, H., Goutard, F., Duboz, R., Bisschop, S.P.R. and Thompson, P.N. 2011(poster). Poultry market contact networks in Ethiopia: implications for disease spread and surveillance. International Conference on Animal Health Surveillance (ICAHS). Lyon, France, 17-20 May 2011. *Epidemiologie et Sante Animale,* 59-60*:* 380-382.

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