COLLEGE OF HEALTH SCIENCES,

SCHOOL OF PUBLIC HEALTH,

UNIVERSITY OF GHANA, LEGON

DOMESTIC DUCKS AS POTENTIAL RESERVOIR OF AVIAN INFLUENZA VIRUS IN POST HPAI H5N1 OUTBREAK AREA, SUNYANI MUNICIPALITY, BRONG AHAFO REGION

By

VITUS BURIMUAH (DVM)

THIS THESIS IS SUBMITTED TO THE UNIVERSITY OF GHANA, LEGON IN PARTIAL FULFILMENT OF THE REQUIREMENT FOR THE AWARD OF MASTERS OF PHILOSOPHY IN *APPLIED EPIDEMIOLOGY* AND DISEASE CONTROL DEGREE

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DEPARTMENT OF EPIDEMIOLOGY AND DISEASE CONTROL
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DECLARATION

This work is the result of an independent investigation under the supervision of Dr. William Kwabena Ampofo and Professor Bawa Awumbila. Where my work is indebted to the works of others, I have made acknowledgements. I declare, therefore that this work has not been accepted in substance for other degree, nor is it concurrently being submitted in candidature for any other degree.

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DEDICATION

This peace of academic work is dedicated to my dear mum, Mrs. Marcela Burimuah (of blessed memory) - my source of inspiration, my dad, Mr. Sylvinus Burimuah and my two sisters, Christina and Grace.

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This work would not have been possible without the support and guidance of my supervisors; Dr. William Ampofo of the Noguchi Memorial Medical Research Institute (NMMRI) and Prof. Bawa Awumbila of the Animal Science Department, both of the University of Ghana. I am grateful to both of them particularly Dr. William Ampofo, my primary supervisor for the provision of reagents and other laboratory logistics from the National Influenza Centre which is supported by the U.S MAMRU-3, CDC, WHO and US DoD.

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LIST OF ABBREVIATIONS

AI	Avian Influenza
AIV	Avian Influenza virus
^{O}C	Degree Celsius
CDC	Centres for Disease Control and Prevention
Ct	Cycle Threshold
DEFRA	Department for Environment, food and Rural Affairs
DIVA	Differentiating Vaccinated from Infected Animals
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
ELISA	Enzyme Linked Immunosorbent Assay
EU	European Union
FAO	United Nations Food and Agriculture Organization
HA	Haemagglutinin
HI	Haemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
HPAIV	Highly Pathogenic Avian Influenza Virus
LPAIV	Low Pathogenic Avian Influenza Virus
µl	micro liter
ml	milliliters
NA	Neuraminidase
NMIMR	Noguchi Memorial Institute for Medical Research
OIE	International Organization for Epizootic
RNA	Ribonucleic Acid
RRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
RT-PCR	Reverse Transcriptase Polymerase Chain Reaction
SARS	Severe Acute Respiratory Syndrome

USAID	United States Agency for International Development
VSD	Veterinary Services Directorate
WHO	World Health Organization

ABSTRACT

BACKGROUND

From July 2009 to October 2010, we conducted a cross sectional study in ducks in nine randomly selected farms in the Sunyani Municipality. The study was done to determine whether the AI virus infection that was reported in the area in May 2007, was circulating silently in ducks or not. The sample size was calculated using Epi info version 3.4.1 at 95% confidence level, absolute precision of 5% and assuming 0.5 prevalence of Avian Influenza A virus in ducks in the study area using the formula $N=z^2 p (1-p)/d^2$.

Approval for the study was obtained from the Department of Epidemiology and Disease Control of the School of Public Health and the Scientific Technical Committee of NMIMR. A written informed consent was obtained from ducks farmers and other stakeholders.

Samples collection was done simultaneously with questionnaire administration to farmers. We collected a total of 526 samples made up of 384 cloacal swabs and 142 feather tissues from 384 ducks. Samples were collected from a commercial duck farm, seven backyard holdings and one live birds market in six randomly selected communities in the Sunyani Municipality. All the 526 samples were duly processed and subjected to Influenza Type A Matrix Gene analysis using RRT-PCR.

All samples tested were negative for Influenza Type A viruses. However, we observed that biosecurity practices which are key to reintroduction of the virus in the area were not adhered to in 89 %(8/9) of the sites investigated. Our finding revealed that only the commercial farm investigated in this study complied with fifteen (78.9%) of the nineteen different farm practices observed.

There was no evidence that domestic ducks in the study area are serving as silent *reservoir of Influenza Type A viruses*. However, if nothing is done to immediately stop the increasing deterioration of bio-security and farm management practices, reintroduction of the AI virus into the area is just a matter of time. Therefore, there is the need for continuos surveillance and education of stakeholders on standard bio-security and farm management *practices in the area*.

CHAPTER ONE

INTRODUCTION

1.1 Background

Avian Influenza (AI) is an infectious disease of birds caused by influenza A viruses. Migratory waterfowls - most notably wild ducks - are the natural reservoirs of all influenza A viruses (Hinshaw and Webster, 1982; Webster *et al.*, 1992; Stallknecht and Brown 2007).

There are 16 main subtypes of influenza A viruses, of which strains within the H5 and H7 subtypes cause Highly Pathogenic Avian Influenza (HPAI), which is highly contagious and rapidly fatal resulting in nearly 100% mortality in infected domestic flocks (Center for Infectious Diseases Research and Policy, 2007).

Recently, avian influenza has acquired world-wide attention when a highly pathogenic strain of the subtype H5N1, which probably arose before 1997 in Southern China, gained epizootic status in poultry throughout South East Asia. The H5N1 virus had traversed interclass barriers (Perkins and Swayne, 2003) and had been transmitted from birds to mammals (cats, swine, and humans). Although not an entirely unprecedented event (Koopmans, 2004, Hayden and Croisier, 2005), the substantial number of documented cases in humans, associated with severe disease and several fatalities raised serious concerns about a pandemic potential of the H5N1 strain (Klempner and Shapiro, 2004; Webster, 2006). There are several further lines of evidence suggesting that the H5N1 virus has acquired increased pathogenic potency

for several mammalian species. Justifiably, this has caused world-wide public concern (Kaye and Pringle, 2005).

Chickens and turkeys are particularly susceptible to epidemics; direct or indirect contact of domestic flocks with wild waterfowl has been implicated as a frequent cause ((Alexander, 1982; Hinshaw and Webster; 1982, Beard, 1989; Webster *et al* 1992; Easterday, *et al* 1997; Stalknecht and Brown, 2007). Birds that survive infection may excrete virus for up to 10 days, orally and in faeces, thus facilitating further spread (Olsen *et al.*, 2005; Swayne and Beck, 2005). Suspicions that birds may be carrying highly pathogenic virus along their migratory routes were underscored following the detection of outbreaks in wild and domestic birds in the Russian Federation and adjacent parts of Kazakhstan in late August 2005 (WHO, Global Alert and Response). In October 2005, outbreaks were detected in Turkey, Romania, and Croatia (OIE, 2005). These areas lie along the flyways of migratory birds and spread to other areas was considered highly likely.

Most avian influenza viruses affecting humans have caused mild respiratory symptoms or conjunctivitis, with one important exception: the H5N1 strain. The H5N1 strain has caused severe disease throughout the world with high fatality rates starting from 1997, till date. Studies comparing virus samples overtime show that H5N1 has become progressively more pathogenic for mammals, and is now hardier than in the past, surviving several days longer in the environment (Olsen *et al.*, 2005; Beck, 2005; EC, 1992 amended 2004). In 2004, H5N1 caused fatal disease in naturally infected large felines (tigers and leopards) and experimentally infected domestic cats - species not previously considered susceptible to disease caused by any influenza A virus. Several mutations in the virus have been detected during 2005, but

the significance of these mutations in terms of virulence and transmissibility in humans is not fully understood.

In 1997, there was an outbreak of AI in Southern China (Hong Kong) that affected 1.4 million chickens and eighteen people of which six died (Kaye and Pringle, 2005). With the assumption that the human infection came from poultry, all birds were destroyed. Five years after the 1997 China outbreak, the disease resurfaced again in China and by 2005 most Asian countries had experienced outbreaks although they were successfully managed. In spite of these successes in control, the AI virus appears to have become endemic in most of the outbreak countries. This situation is similar in African countries that have experienced AI outbreaks including Egypt, South Africa, Nigeria, and Benin.

In April 2007, despite a ban on importation of poultry and poultry products from HPAI H5N1 affected countries (mostly Southeast Asian countries) and several biosecurity measures enforced by the Government of Ghana, the first outbreak of H5N1 was reported in Ghana by VSD at a small – scale poultry farm at Kakasunanka, near Michel Camp in the Tema Metropolis (April 24, 2007). Subsequently, outbreaks of AI were reported in Sunyani in the Brong Ahafo region (May 15, 2007) and Aflao in the Volta region the same year (June 13, 2007). To date, the reasons for the emergence and spread of the disease in the country have not been determined.

As a result of these outbreaks, the Government of Ghana as well as affected poultry farmers incurred huge economic losses in the form of: compensation to poultry farmers whose birds were affected, cost of equipment and disinfectants, cost of human

resources to control the outbreak and losses in international trade as per the ban on poultry and poultry products.

The compensation for AI ranged from 70% to 90% of the market prices for day-old chicks, broilers, cockerels and layers, while table and fertile eggs were paid at rates of 50% and 60% of market prices respectively. In all, a total of 13,391 birds were affected and 36,376 birds including over six hundred ducks were destroyed. The Government paid an amount of 160,000 US dollars as compensation to affected farmers (VSD Annual Reports, 2007 and 2009). The cost of veterinary interventions and public education on prevention and control of the disease was estimated at 2 million US dollars. Much of this financial support came from donor partners including USAID and FAO (VSD Annual Report, 2009).

Unlike chickens, some domestic ducks are known to be resistant to the viruses and can be asymptomatic carriers of the viruses, thus acting as a "silent reservoir" that perpetuates transmission (Swayne and Beck, 2005 and European Commission (EC), 1992 amended 2004).

The experience of a second outbreak of HPAI in Togo in 2008 after the 2007 outbreak in that country has shown the ability of H5N1 virus to persist discreetly among traditional farms (scavenging poultry) where chicken mortality is common and usually go unreported (anonymous). Although not confirmed in Togo, the role of ducks and related species in the viral circulation of avian influenza was reported in many countries including Nigeria in the 2008 (Alice F *et al.*, 2008).There is therefore the need to investigate the status of avian influenza virus infections in domestic ducks in

the Sunyani Municipality where HPAI occurred in the past. Also, there is a large influx of poultry products from neighbouring Cote d'Ivoire into Ghana via Sunyani.

1.2 PROBLEM STATEMENT AND JUSTIFICATION FOR THE STUDY

The ongoing outbreaks of H5N1 Highly Pathogenic Avian Influenza in some parts of Asia, Africa and Europe, is still posing a real public health threat as HPAI can occasionally infect humans. Ducks play a major role in the epidemiology of avian influenza because wild waterfowls constitute the natural reservoirs of subtypes of influenza A virus.

Some duck species can shed and spread virus from both the respiratory and intestinal tracts while showing few or no disease signs. While the HPAI H5N1 viruses are 100% lethal for chickens and other gallinaceous poultry, the absence of disease signs in some duck species has led to the concept that ducks are the "Trojan horses" of H5N1 in their surreptitious spread of the virus.

In Ghana, domestic ducks may be acting as silent reservoirs for the H5N1 HPAI virus. The risk is greatest in post outbreak areas, where traditional free-ranging ducks, chickens and wildlife mingle frequently sharing the same source of water. This situation prevails in the Sunyani Municipality.

The experience of a second outbreak of HPAI in Togo in 2008 has shown the ability of H5N1 to persist discreetly among traditional farms (scavenging poultry) where chicken mortality is common and usually go unreported (anonymous). Although not confirmed in Togo, the role of ducks and related species in the viral circulation of

avian influenza was reported in many countries including Nigeria (Alice F *et al., 2008*).

The role of domestic ducks in the maintenance and spread of H5N1 and other influenza A viruses has not been investigated in Ghana, though countries such as China, Nigeria and others that have experienced outbreaks of HPAI have conducted such studies.

In Ghana, outbreaks of H5N1 were recorded in 2007 in the Sunyani Municipality (New Dormaa area) of the Brong Ahafo region. The region shares border with La Cote d'Ivoire, a country which reported AI outbreaks in 2006. Brong Ahafo region is also notable for its significant poultry production and active cross border trading activities.

Hence, there is the need to investigate the status of AI infection in ducks in the Sunyani Municipality to determine the presence of any circulating AI strains to guide the adoption of relevant control strategies three years after HPAI outbreaks in this area.

1.3 RESEARCH QUESTIONS

1. Are domestic ducks in the Sunyani Municipality serving as silent reservoirs of the AI virus that was detected in poultry in the area in May, 2007?

2. Are the biosecurity processes and procedures for HPAI in Sunyani Municipality adequate?

1.4 STUDY OBJECTIVES

1.4.1 Main Objective

- To determine if the H5N1 virus is circulating in ducks in the Sunyani Municipality

1.4.2 Specific Objectives

- To determine the prevalence of AI virus in ducks population in the Sunyani Municipality
- To characterize AI viruses possibly circulating in the Sunyani Municipality
- To investigate the application of molecular methods for AI detection in cloacal swabs and feather tissues
- To assess adherence to bio-security measures in ducks farms in the Sunyani Municipality

CHAPTER TWO

LITERATURE REVIEW

2.1 THE INFLUENZA VIRUS

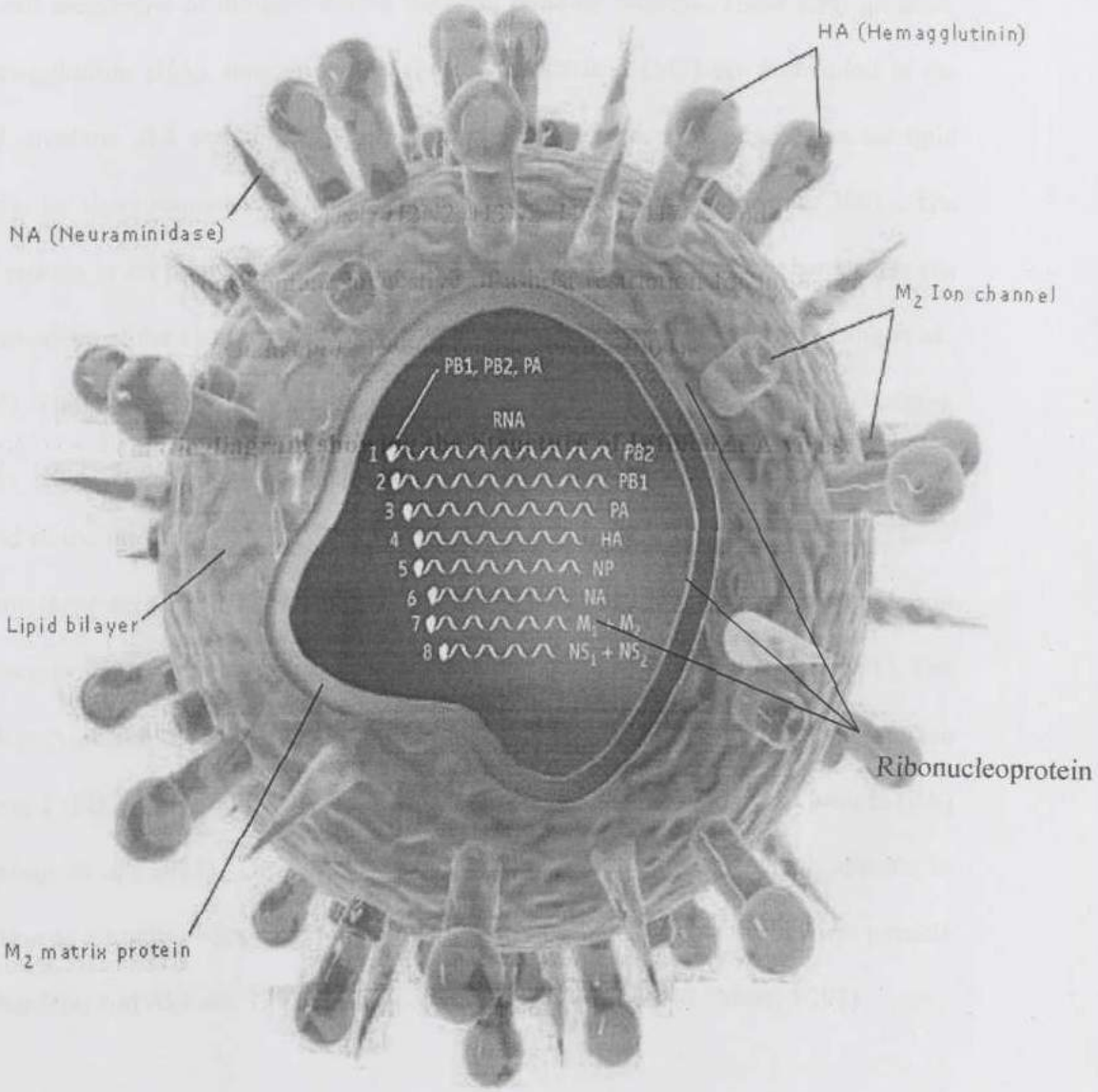
Influenza viruses are the etiological agents of influenza (Smith, Andrewes, and Laidlaw, 1933). Influenza viruses are classified within the viral family *Ortho myxoviridae* from the Greek words *orthos*, meaning "standard, correct," and *myxa*, meaning "mucus" (Cheung and Poon, 2007) which is characterized by a segmented negative sense stranded RNA genome (Shin-Ru *et al.*, 2005; Webster *et al.*, 1982; Rohm *et al.*, 1996). There are four genera in the family of *Orthomyxoviridae*: Influenza virus A, Influenza virus B, Influenza virus C, and Thogotovirus (Cheung and Poon, 2007).

2.2 CLASSIFICATION OF INFLUENZA VIRUSES

Influenza A, B and C viruses can be distinguished based on the antigenic differences between their nucleoproteins (NP) and matrix (M) proteins (Lamb and Krug, 2001). *Influenza A and B viruses contain* 8 RNA genomic segments, whereas influenza C virus contains only 7 RNA genomic segments (Desselberger *et al.*, 1980). All of these viruses can naturally infect humans. However, only influenza A virus has been responsible for all influenza pandemics (Potter, 1998). *Influenza A viruses are further* subdivided into different subtypes based on the antigenic variation of the haemagglutinin (HA) and neuraminidase (NA) surface glycoproteins (Cheung and Poon, 2007). Currently, there are 16 known subtypes of HA (Fouchier *et al.*, 2005) and 9 known subtypes of NA (Laver *et al.*, 1984). Each of these subtypes can be isolated from aquatic birds; this suggests that avian species are the natural hosts of

influenza viruses, while only H1N1, H2N2, H3N2, H5N1, H7N7 and H9N2 *subtypes* have been isolated from human, suggestive of a host restriction for influenza viruses (Subbarao *et al.*, 1998; Guan *et al.*, 1999; Fouchier *et al.*, 2004).

Figure 1: Schematic diagram showing the Structure of Influenza A virus.



Source: *Science* Vol. 312, pp. 380.

The influenza virus enveloped with pleomorphic virions of shapes ranging from small spherical to long filamentous forms. Several viral proteins; HA, NA, M1 and M2 are known to have effects on the morphology of the influenza virus particles (Burleigh *et al.*, 2005; Elleman and Barclay, 2004; Enami *et al.*, 1996; Mitnaul *et al.*, 1996; Roberts *et al.*, 1998). The Influenza A viral particle has a lipid envelope; derived from the cell membrane of the host during the viral budding process. Three viral proteins, haemagglutinin (HA), neuraminidase (NA) and matrix 2 (M2) are embedded in the lipid envelope. HA and NA are spike glycoproteins and they are attached to the lipid bilayer by short sequences of hydrophobic amino acids (Lamb and Krug, 2001). The M2 protein is an integral membrane protein that functions as an ion channel for the acidification of the viral particle during viral infection (Pinto *et al.*, 1992; Wang *et al.*, 1994). There is a matrix 1 (M1) protein layer under the viral lipid envelope (Ruigrok *et al.*, 1989). Inside the virion, all eight viral ribonucleic acid (vRNA) segments are bound to the nucleoprotein (NP) and to the influenza virus RNA polymerases to form ribonucleoprotein (RNP) complexes (Lamb and Choppin, 1983). Each NP monomer interacts with approximately 20 nucleotides of the vRNA (Lamb and Krug, 2001). The RNA polymerase complex is made up of three polymerase subunits: basic polymerase protein 2 (PB2) basic polymerase protein 1 (PB1) and acidic polymerase protein (PA) (Jennings *et al.*, 1983). The non-structural protein 2 (NS2) protein which appears to function as a nuclear export protein is also present in low amounts in the viral particle (Richardson and Akkina, 1991; Yasuda, *et al.*, 1993; O'neill and Palese, 1998).

2.3 GENOMIC ORGANIZATION OF THE INFLUENZA VIRUS

According to Palese 1997, influenza A virus genome consists of 8 segments as follows:

1. Segment 1 (PB2)

Segment 1 encodes one of the influenza viral polymerase subunits basic polymerase protein 2 (PB2) (Huang, *et al.*, 1990; Perales and Ortin, 1997). Studies have shown that PB2 contains a nuclear localization signal and is transported into the nucleus of infected cells for viral transcription and replication (Jones *et al.*, 1986; Mukaigawa and Nayak, 1991; Perales *et al.*, 1996,). Studies have also shown that the PB2 subunit is a cap binding protein (Ulmanenen *et al.*, 1981; Blass *et al.*, 1982; Ulmanen *et al.*, 1983) and also has endonuclease activity, using host mRNA to generate cap primers for viral mRNA synthesis (Krug *et al.*, 1979; Plotch *et al.*, 1979; Bouloy *et al.*, 1980; Plotch *et al.*, 1981; Shi *et al.*, 1995).

2. Segment 2 (PB1)

Segment 2 encodes the basic polymerase protein 1 (PB1). Evidence suggests that PB1 is an RNA polymerase and plays a key role in the assembly of three polymerase protein subunits and RNA polymerization (Cheung and Poon, 2007).

3. Segment 3 (PA)

The acidic polymerase protein PA is encoded by segment 3. It is the smallest subunit of the influenza RNA polymerase complex (Cheung and Poon, 2007). It possesses nuclear localization signals (Nieto *et al.*, 1994) required for transport into the nucleus

of infected cells (Jones *et al.*, 1986). PA is also known to play a key role in viral transcription and replication (Perales and Ortin, 1997).

4. Segment 4 (HA)

Segment four encodes the haemagglutinin (HA) protein which is a glycoprotein containing 2 of 3 glycosylation sites, with a molecular weight of approximately 76,000. It spans the lipid membrane so that the major part, which contains at least 5 antigenic domains, is presented at the outer surface. The HA protein serves as a receptor by binding to sialic acid (N-acetylneuraminic acid) and induces penetration of the interior of the virus particle by membrane fusion (Steinhauer and Wharton, 1998; Lamb and Krug, 2001,). The haemagglutinin is the main influenza virus antigen, the antigenic sites being A, B (carrying the receptor binding site), C, D, and E and is therefore the major target for neutralizing antibodies (Staudt and Gerhard, 1983).

5. Segment 5 (NP)

Segment 5 encodes the nucleoprotein (NP) which is an essential component required for transcription and replication (Huang *et al.*, 1990; Perales and Ortin, 1997).

6. Segment 6 (NA)

Segment 6 encodes the neuraminidase NA protein which is a surface glycoprotein consisting of a box-shaped globular head, a thin stalk, a transmembrane domain and a cytoplasmic domain (Air and Laver, 1989). The NA protein acts as an enzyme, cleaving sialic acid from the HA molecule, from other NA molecules and from glycoproteins and glycolipids at the cell surface. It serves as an important antigenic

site, and in addition, seems to be necessary for the penetration of the virus through the mucin layer of the respiratory epithelium (Mitnaul *et al.*, 1996). The NA protein carries several important amino acid residues in which, if mutatation occurs, can lead to resistance against neuraminidase inhibitors (Wetherall *et al.*, 2003).

7. Segment 7 (M1 and M2)

Segment 7 encodes two proteins, Matrix proteins 1 and 2; M1 and M2 (Shih *et al.*, 1995).

M1 protein: Separates the ribonucleoprotein from the viral membrane (Ruigrok *et al.*, 1989). It interacts with both vRNA and protein components of RNP in assembly and disassembly of influenza A viruses (Ye *et al.*, 1999). The M1 protein binds to the cell membrane (Gregoriades and Frangione, 1981) and seems to have an effect on viral assembly, budding and viral morphology (Enami *et al.*, 1996; Roberts *et al.*, 1998; Gomez-Puertas *et al.*, 2000; , Liu, *et al.*, 2002; Elleman and Barclay, 2004).

M2 protein: It is an integral membrane protein (Bui *et al.*, 1996) and has ion channel activity for pH regulation (Ciampor *et al.*, 1992; Pinto *et al.*, 1992; Wang *et al.*, 1994). In the endosome of infected cells, the ion channel activity of M2 allows acidification of the interior of the incoming particle (Cheung and Poon, 2007). This acidification is essential for viral replication by helping with dissociation of viral ribonucleoproteins from M1 proteins for nuclear import (Martin and Helenius, 1991; Bui *et al.*, 1996). The M2 ion channel activity also maintains a high pH in the Golgi vesicles so as to stabilize the native conformation of newly synthesized haemagglutinin HA during intracellular transport for viral assembly (Takeuchi and Lamb, 1994).

8. Segment 8 (NS1 and NS2)

It encodes 2 proteins non-structural proteins 1 and 2, NS1 and NS2 (Alonso-Caplen and Krug, 1991; Nemeroff *et al.*, 1992).

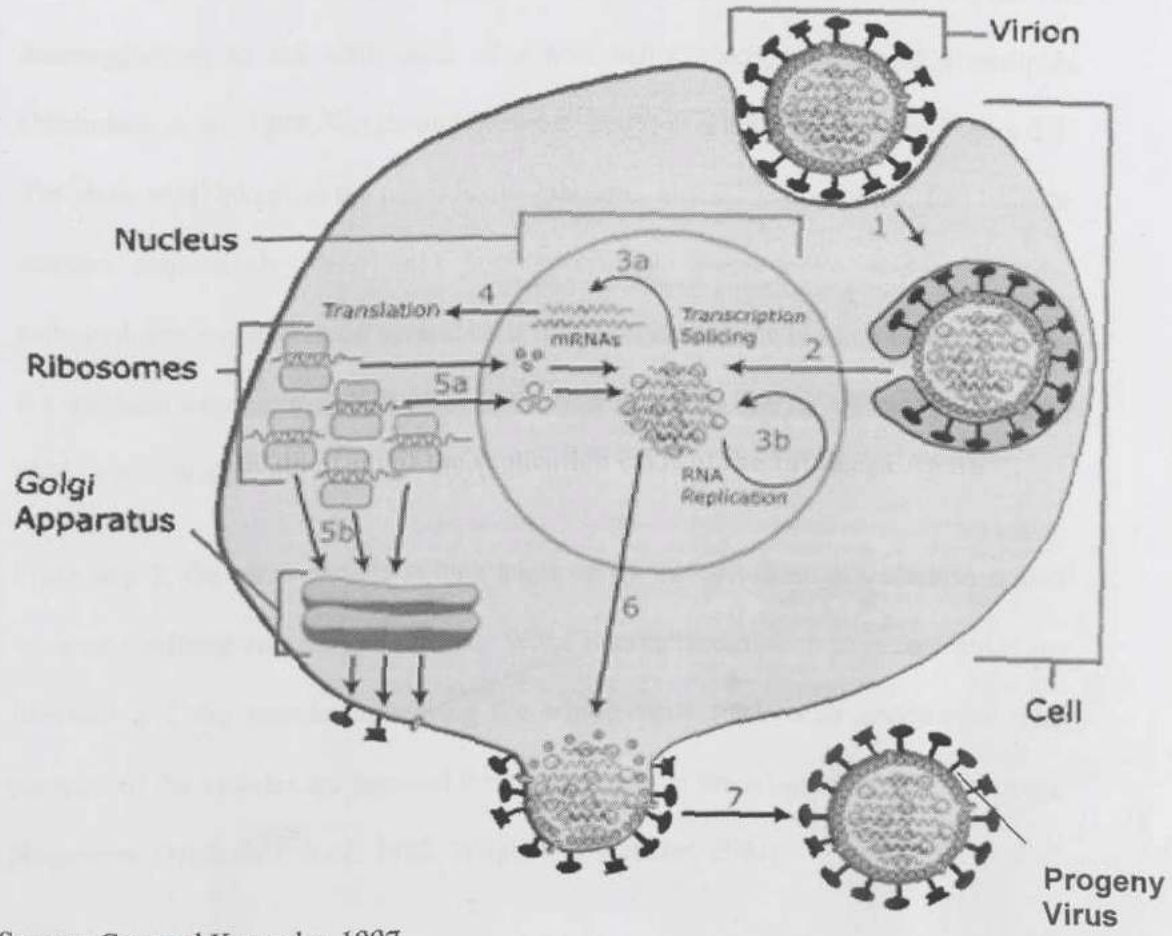
NS1: It is the only non-structural protein of the Influenza virus (Cheung and Poon, 2007). Its function is to regulate viral protein expression by binding to different RNA molecules such as poly (A)-containing cellular RNA (Qiu and Krug, 1994); vRNA (Hatada *et al.*, 1992) vRNP (Marion *et al.*, 1997b) double stranded RNA (Hatada and Fukuda, 1992) and small nuclear RNA (Qiu *et al.* 1995).

NS2: There is evidence that NS2 protein contains a nuclear export signal and facilitates the viral ribonucleoprotein export (O'neill, 1998). The NS2 protein was initially described as a non structural protein (Cheung and Poon, 2007) but recent studies have reported that NS2 is incorporated into viral particles in low amounts (Richardson and Akkinna, 1991).

2.4 INFLUENZA VIRUS REPLICATION

The influenza virus genome has negative-sense RNA. However, unlike other negative-sense single stranded RNA viruses, the transcription and translation site for the influenza virus is in the nucleus of an infected cell (Palese, 1977; Hertz *et al.*, 1981; Jackson *et al.*, 1982). All eight RNA segments of the virus have the first 12-13 nucleotides at their 3′ and 5′ ends conserved. Each segment also contains 2 to 3 segment specific nucleotides near each end. These RNA sequences are partially complimentary and can form a pan handle structure (Robertson, 1979; Skehel *et al.*, 1978).

Figure 2: Schematic diagram of the replication cycle of the Influenza A virus



Source: Cox and Kawaoka, 1997.

Figure 2: Stages of the replication cycle of *Influenza A virus*

1. Entry of viral particle into the cell
2. Uncoating of viral particle

3 a and b: Transcription / Replication in the nucleus

4. RNA transported into cytoplasm for translation into viral proteins

5 a. Viral proteins transported back into the nucleus

5 b. Secretion of HA and NA in the Golgi apparatus

6. Assembly of progeny virus

7. Budding off of progeny virus from plasma membrane of infected cell.

2.4.1 Adsorption, entry and uncoating of virus particle

The influenza virus binds to the host cell surface by fixing the outer top of the haemagglutinin to the sialic acid of a host cell's glycoproteins and glycolipids (Nicholson *et al.*, 1998; Wright and Webster, 2001) as shown in step 1 of Figure 2.2. The sialic acid linkage to the penultimate galactose, alpha 2, 3 or alpha 2, 6 in birds or humans respectively, determines host specificity. Since sialic acid- presenting carbohydrates are present on several cells of the organism, the binding capacity of the HA explains why multiple cell types in the host organism can be infected (Nicholson *et al.*, 1998; Wright and Webster, 2001).

From step 2; the virus particle is then taken up by the cell through a clathrin-coated receptor mediated endocytosis process. When internalized, the clathrin molecules are liberated and the vesicle harbouring the whole virus fuses with endosomes. The contents of the vesicles are digested through a stepwise lowering of the pH within the phagosome (Nicholson *et al.*, 1998; Wright and Webster, 2001).

The lowering of the pH is stopped by the action of the M2 protein which induces the partial liberation of the fusion peptide of the HA. This allows the fusion of the HA with the membrane of the vesicle and liberation of the ribonucleoproteins into the cytoplasm. The influx of ions from the endosome to the virus particle leads to disconnection of the different viral proteins; M1-protein aggregation is disrupted and ribonucleoproteins no longer adhere to the M1-protein complex. Uncoating is completed within 20 - 30minutes of virus attachment to the cell (Nicholson *et al.*, 1998; Wright and Webster, 2001).

2.4.2 Transcription

Transcription of mRNA takes place in the nucleus of an infected cell as illustrated in step 3 of figure 2.2. It is initiated by a capped RNA fragment, which is cleaved from host mRNA by a cap snatching mechanism (Braam *et al.*, 1983; Krug *et al.*, 1979; Plotch *et al.*, 1979). The PB2 polymerase subunit binds to the 5′ end of the host cell mRNA and cleaves it about 10-15 nucleotides downstream from the cap structure after predominantly an A or G residue (Plotch *et al.*, 1981). This cleavage requires a methylated cap structure on the RNA substrate (Bouloy *et al.*, 1980). The endonuclease activity of PB2 is stimulated by vRNA (Cianci *et al.*, 1995; Hagen *et al.*, 1994; Li *et al.*, 1998). The viral polymerase complex thus uses the host mRNA for viral mRNA synthesis (Shih and Krug, 1996). The short capped oligonucleotide obtained after endonuclease cleavage is used by the viral polymerase as a primer for transcription. Initiation of viral transcription requires the interaction between the 5' and 3' conserved sequences of vRNA (Luo *et al.*, 1991) and it is terminated at a track of 5-7 U residues approximately 17 nucleotides from the 5' end of vRNA. A poly A tail is then added to the mRNA transcript and this polyadenylation is known to be a host – independent process (Poon *et al.*, 1998; Plotch and Krug, 1977).

2.4.3 Synthesis of cRNA

Though the mechanism for the switching from transcription to replication is still poorly understood, it is generally accepted that the first step in replication is the production of positive strand copies cRNA of each segment. cRNA is a full length copy of the vRNA and can be used as a template for genomic RNA synthesis. cRNA

is different from products of transcription in that the 5′ ends are not capped. Again cRNA is not polyadenylated (Hay *et al.*, 1982).

2.4.4 Expression of Influenza Viral Proteins

The replication and transcription of the Influenza viral genome is a highly selective process (Smith and Hay, 1982) as illustrated in steps 5 of figure 2.2. Immediately after infection, primary transcription occurs and here all eight mRNAs are synthesized in equal amounts (Hay *et al.*, 1977). This is followed by the second transcription stage which can be further subdivided into early and late stages. First, NS1 and NP vRNA are synthesized and become the predominant viral proteins in infected cells at this stage (Hay *et al.*, 1977). During the late stage, the NS1 protein synthesis is reduced and HA, NA and M1 mRNAs are expressed (Shapiro *et al.*, 1987). Most of the capped and polyadenylated viral mRNAs are transported from the nucleus into the cytoplasm for protein synthesis. Membrane-bounded proteins, such as HA, NA, and M2 use the secretory pathway of the *trans*-Golgi network for protein maturation. HA and NA proteins are post-translationally modified and transported to the cell surface for integration into the cell membrane (Shapiro *et al.*, 1987).

2.4.5 Virus Assembly and release of Progeny Virus

Generally, the RNPs are transported to the nucleus where the polymerase complex binds to viral RNA, cleaves the viral RNA by its endonuclease activity and simultaneously leads to elongation. The production of vRNA is limited by the NP in favour of mRNA and both are transported to the cytoplasm where viral proteins are generated at the ribosome. Part of the viral mRNA is spliced by cellular enzymes so that finally viral proteins such as M1 and NS2 can be synthesized without any further

cleavage. Some of the newly synthesized viral proteins are transported to the nucleus where they bind to viral RNA to form RNPs. Other newly synthesized viral proteins are processed in the endoplasmic reticulum and the Golgi apparatus where glycosylation occurs. These modified proteins are transported to the cell membrane where they stick in the lipid bilayer. Different viral components are transported to the lipid bilayer differently. The nucleocapsids are assembled in the nucleus and then move to the cell surface. HA and NA are synthesized in the endoplasmic reticulum and transported to the lipid bilayer. Finally when the desired concentration is reached at the plasma membrane; RNPs and M1 proteins aggregate and condenses to produce a viral particle. The particle is extruded from the membrane and is liberated by neuraminidase activity (Wright and Webster, 2001). New progeny viruses are produced within 8 - 10hrs. This is shown in steps 6 and 7 of figure 2.

2.4.6 Shedding of the Virus and Infectivity

Immunohistological pictures show that foci of virus-producing cells are clustered in the mucous layer of the respiratory tract, in the gut and even in endothelial layers, myocardium and brain. Within nasal secretions, millions of virus particles per ml are shed, so that a 0.1 µl aerosol particle contains more than 100 virus particles. A single human infectious dose of influenza virus might be between 100 and 1,000 particles. At least during the early course of influenza infection, the virus can be found also in the blood and in other body fluids (Wright and Webster, 2001).

2.4.7 Stability of Influenza Virus

Infectivity of influenza virus particles is preserved depending on temperature, pH and salinity of the water, and ultraviolet irradiation. At 4°C, the half-life of infectivity is

about 2-3 weeks in water. Due to the conformation of the lipid bilayer, survival under normal environmental conditions should be shorter. Infectivity of the influenza virus particle is easily inactivated by all alcoholic disinfectants, chlorine and aldehydes. As far as it is known, temperatures above 70°C will destroy infectivity in a few seconds (Wright and Webster, 2001).

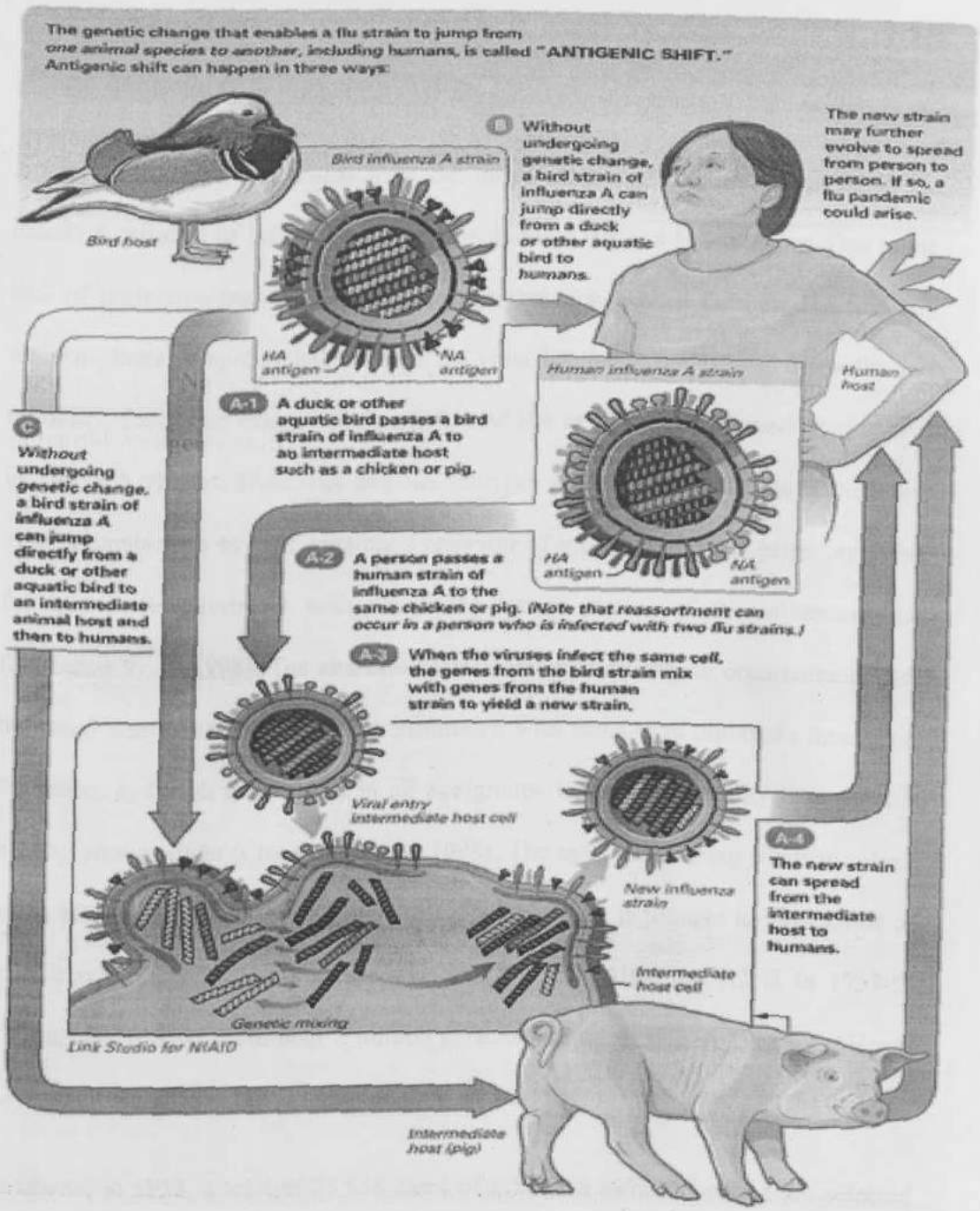
2.5 *Antigenic Drifts*

The two surface antigens HA and NA of the influenza virus undergo antigenic variation independent of each other (Lamb and Krug, 2001). Minor antigenic changes called antigenic drift are due to the low fidelity of the viral polymerase as well as host immune selection leading to the accumulation of point mutations in the HA and NA genes, resulting in amino acid sequence changes in the protein (Shin-Ru, *et al.*, 2005). These point mutations occur unpredictably and result in minor changes to these surface proteins. Antigenic drift produces new virus strains that may not be recognized by antibodies to earlier influenza strains. This process works as follows: a person infected with a particular influenza virus strain develops antibody against that strain. As newer virus strains appear, the antibodies against the older strains might not recognize the "newer" virus, and infection with a new strain can occur. This is one of the main reasons why global surveillance is critical in order to monitor the evolution of human influenza virus.

2.6 Antigenic Shifts

Antigenic shift is a more drastic type of genetic change and is due to abrupt mutations in the sequence of the viral surface antigens resulting in the sudden emergence of a completely new subtype of virus (Webster, *et al.*, 1982). These changes are too extreme to be explained by mutations generated by faulty viral polymerase, therefore they are thought to be the result of genetic reassortment between human and influenza viruses from other animals including birds and swine. Antigenic shift can occur either through direct animal (poultry)-to-human transmission or through mixing of human influenza A and animal influenza A virus genes to create a new human influenza A subtype virus. A global influenza pandemic (worldwide spread) may occur if three conditions are met: A new subtype of influenza A virus is introduced into the human population, the virus causes serious illness in humans and the virus can spread easily from person to person in a sustained manner. Influenza B and C viruses do not appear to exhibit antigenic shift (Wright and Webster, 2001).

Figure 3: The National Institute of Allergy and Infectious Disease illustration of potential Antigenic shift.



Source: NIAID, illustration of potential influenza antigenic shift, 2009.

2.7 INFLUENZA EPIDEMICS AND PANDEMICS

The influenza virus is known for its unique ability to cause recurrent epidemics and global pandemics during which acute febrile respiratory illness occurs in all age groups. Antigenic drifts are known to cause seasonal epidemics (Juckett, 2006).

An influenza pandemic results from the introduction and subsequent spread in the human population of Influenza A viruses with novel HA and NA subtypes. Due to the lack of protective immunity in the human population against the new HA and NA antigens, there is rapid global spread of the virus, leading to widespread morbidity and mortality. Pandemic strains often contain new HA or NA genes derived from animal influenza A viruses. These HA and NA subtypes are known to circulate in birds and other animals such as pigs, creating a reservoir of influenza A virus genes, available for genetic reasssortment with circulating human strains of the influenza virus (Subbarao, *et al.*,1998). The characteristics of pandemics include occurrence outside the usual season, extremely rapid transmission with concurrent outbreaks throughout the globe, and high attack rates in all age groups with high mortality rates even in healthy young adults (Cox & Kawaoka 1998). The most devastating pandemic strain is the H1N1 virus in 1918-19 ("Spanish flu"). This virus is thought to have killed up to 100 million persons, (Taubenberger, *et al.*, 2001). Also, the H2N2 in 1957-58 ("Asian flu") killed more than 2 million persons and the H3N2 in 1968-69 ("Hong-Kong flu") which also killed 1 million persons worldwide (Luke, *et al.*, 2006).

In Ghana, in 1973, a total of 23,858 cases of influenza were recorded from selected health centres in the greater Accra region between the period of 1st October 1973 to 30th December 1973 (Nowacki and Addy, 1975). The conclusion was drawn that this

epidemic was caused by Influenza A/Hong Kong/1/68 virus (Addy, Mingle and Nowacki, 1976). Again in 1996, Mingle *et al.*, reported that there was an outbreak of Influenza A (H3N2) in Accra (Mingle *et al., 1996*).

2.8 AVIAN INFLUENZA DISEASE

Avian influenza (AI) is a disease of viral etiology that ranges from a mild or even asymptomatic infection to an acute, fatal disease of chickens, turkeys, guinea fowls, and other avian species, especially migratory waterfowl (Alexander, 1982; Hinshaw and Webster 1982; Beard, 1989; Webster *et al* 1992; Easterday *et al* 1997; Stalknecht and Brown 2007). Wild water birds have been identified as natural reservoir host of avian influenza viruses. Generally, the infection is asymptomatic as influenza A virus strains of low pathogenicity co-exist in almost perfect balance in wild water birds (Webster, 1992 and Alexander, 2000).

Recently, avian influenza has acquired world-wide attention when a highly pathogenic strain of the subtype H5N1, which probably arose before 1997 in Southern China, gained enzootic status in poultry throughout South East Asia. The H5N1 virus had traversed interclass barriers (Perkins and Swayne, 2003) and had been transmitted from birds to mammals (cats, swine, and humans). Although not an entirely unprecedented event (Koopmans, 2004; Hayden and Croisier, 2005), the substantial number of documented cases in humans, associated with severe disease and several fatalities raised serious concerns about a pandemic potential of the H5N1 strain (Klempner and Shapiro 2004; Webster, 2006). There are several further lines of evidence suggesting that the H5N1 virus has acquired increased pathogenic potency

for several mammal species. Justifiably, this has caused world-wide public concern (Kaye and Pringle, 2005).

Infection with avian influenza viruses in domestic poultry causes two main forms of disease that are distinguished by low and high extremes of pathogenicity.

2.9 HIGHLY PATHOGENIC AVIAN INFLUENZA (HPAI)

HPAI viruses belong to the H5 and H7 subtypes exhibiting a multiple-basic amino acid cleavage site at the precursor of the haemagglutinin molecule and therefore sensitive to most enzymes of the body. HPAI is a dead-end infection in certain domestic birds (e.g. Chickens and Turkeys) and has a variable clinical behavior in domestic waterfowls and in wild birds, in which it may or may not cause clinical signs and mortality. To date the potential role as reservoirs of infection of wild birds and waterfowls (ducks) has been described only for the Asian HPAI H5N1 virus. The ecological and epidemiological implications of this unprecedented situation are not predictable.

2.10 LOW PATHOGENICITY AVIAN INFLUENZA (LPAI)

Avian influenza viruses belong to subtypes (H1-H16), lacking the multiple-basic amino acid cleavage site and therefore only sensitive to trypsin-like enzymes, are perpetuated in nature in the wild bird population. Feral birds, particular waterfowl (mostly ducks species) represent the natural hosts for these viruses and are therefore considered an ever-present source of viruses. Following introduction into domestic bird populations, these viruses cause low pathogenic avian influenza (LPAI). This is a localized infection, resulting in a mild respiratory disease, depression and egg

production decrease in laying birds. Current theories suggest that HPAI viruses emerge from H5 and H7 LPAI progenitors by mutation or recombination although there must be more than one mechanism by which this occurs. This is supported by phylogenetic studies of H5 subtype viruses, which indicate that HPAI viruses do not constitute a separate phylogenetic lineage or lineages, but appear to arise from non-pathogenic strains and the *in vitro* selection of mutants virulent for chickens from an avirulent H7 virus. It appears that such mutations occur only after the viruses have moved from their natural wild bird host to poultry. However, the mutation to virulence is unpredictable and may occur very soon after introduction to poultry, or after the LPAI virus has circulated for several months in domestic birds. The scientific evidence collected in recent years, lead to the logical conclusion that not only HPAI viruses must be controlled in domestic populations but also LPAI viruses of the H5 and H7 subtypes, as they represent HPAI precursors. For this reason, both HPAI and LPAI belonging to H5 and H7 subtypes are considered by World Organization for Animal Health (OIE) as "Notifiable Diseases".

2.11 GENESIS OF HIGHLY PATHOGENIC AVIAN INFLUENZA VIRUSES

In wild water birds, LPAI viruses are a natural part of the ecosystem. They have been isolated from over 90 species of wild bird (Stallknecht and Shane 1988; Olsen *et al.*, 2006; Lee, 2008), and are thought to have existed alongside wild birds for millennia in balanced systems. In their natural hosts, avian influenza viruses infect the gastro-intestinal tract and are shed through the cloacae; they generally do not cause disease although some behavioural anomalies have been reported, such as reduced migratory and foraging performance in Bewick's Swans Cygnus *columbianus bewickii* (Van Gils *et al.*, 2007); instead, the viruses remain in evolutionary stasis as indicated by low

genetic mutation rates (Gorman *et al.*, 1992; Taubenberger *et al.*, 2005). When LPAI viruses are transmitted to vulnerable poultry species, only mild symptoms such as a transient decline in egg production or reduction in weight gain (Capua and Mutinelli, 2001) are induced. However, where a dense poultry environment supports several cycles of infection, the viruses may mutate, adapting to their new hosts, and for the H5 and H7 subtypes these mutations can lead to generation of a highly pathogenic form. Thus, HPAI viruses are essentially products of intensively farmed poultry, and their incidence has increased dramatically with the greatly enhanced volume of poultry production around the world (Greger, 2006). In the first few years of the 21st century, the incidence of HPAI outbreaks has already exceeded the total number of outbreaks recorded for the entire 20th century (Greger, 2006). In general, these outbreaks should be viewed as something artificial, made possible by intensive poultry production techniques. After an HPAI virus has arisen in poultry, it has the potential both to re-infect wild birds and to cause disease in various mammalian taxa. If influenza A viruses adapt inside these new hosts to become highly transmissible, there can be devastating consequences, such as the human influenza pandemics of the 20th century (Kilbourne, 2006). The conditions necessary for cross-infection are provided by agricultural practices that bring together humans, poultry and other species in high densities in areas where there is also the potential for viral transmission from infected poultry, poultry products and waste to wild birds, humans and other mammals in shared wetlands and in 'wet' (i.e. live animals) markets (Shortridge, 1977; Shortridge *et al.*, 1977).

2.12 HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 OF ASIAN LINEAGE (HPAI H5N1).

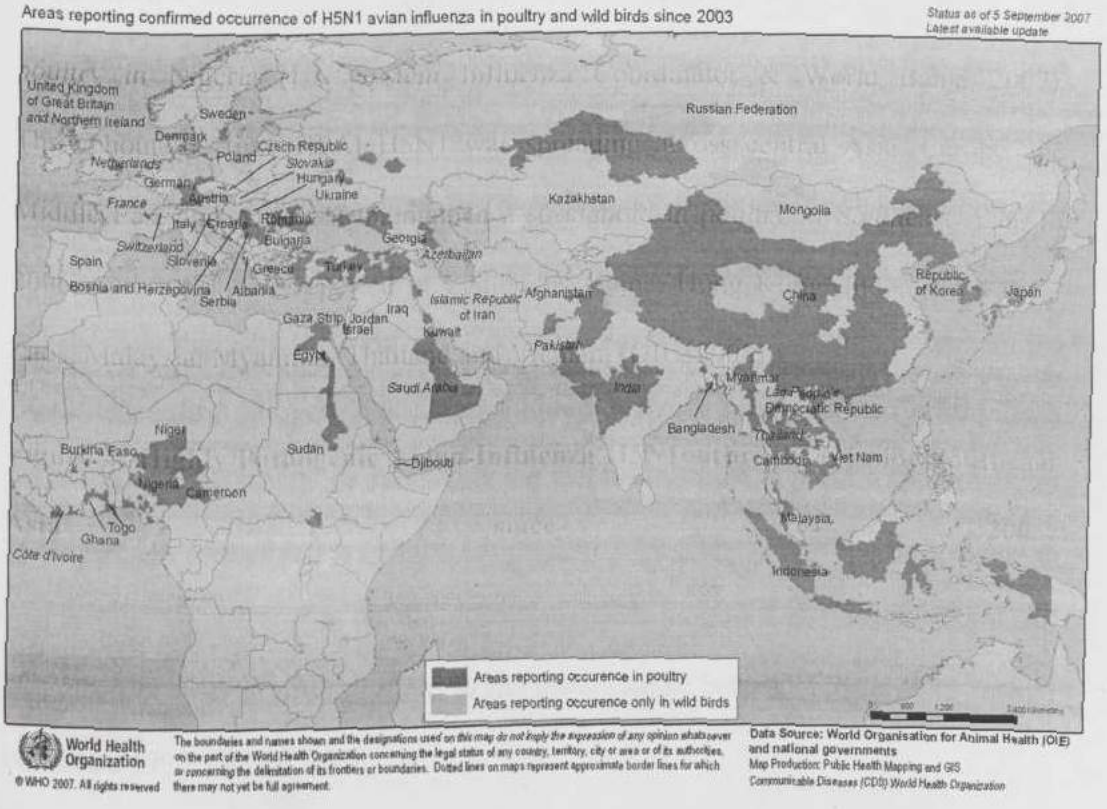
The highly pathogenic avian influenza H5N1 of Asian lineage has infected domestic, captive and wild birds in more than 60 countries in Asia, Europe and Africa (OIE, 2008). By November 2005, i.e. before widespread occurrence in western Eurasia and Africa, over 200 million domestic birds had died from the disease or been destroyed in attempts to control its spread; the economies of the worst affected countries in southeast Asia have suffered greatly, with lost of revenue estimated at over $10 billion (Diouf, 2005), and there have been serious human health consequences. By March 2008, the World Health Organization had confirmed more than 370 human cases, over 60% of those fatal (World Health Organization, 2008). Sporadic deaths in wild birds have been reported since 2002 and the first outbreak involving a large number of wild birds was reported in May 2005, in Qinghai province, China (Chen *et al.*, 2005; Liu *et al.*, 2005). Between 2002 and the present, the virus has infected a wide range of wild bird species (Olsen *et al.*, 2006; USGS National Wildlife Health Center, 2008; Lee, 2008), but which species are important in H5N1 HPAI movement and whether the virus will become enzootic in wild bird populations is still unknown(Brown *et al.*, 2006). The virus has also infected a limited number of domestic, captive and wild mammals, including captive Tigers Panthera *Tigris* and Leopards Panthera *pardus* and domestic pigs in Southeast Asia, and domestic cats and a wild Stone Marten Martes *foina* in Germany. These cases were the result of 'spillover' infection from birds. There is no known reservoir of HPAI H5N1 virus in mammals and there remains no sound evidence that the virus can be readily transmitted from mammal to mammal.

2.13 GEOGRAPHICAL SPREAD OF HPAI H5N1 OUT OF SOUTHEAST ASIA (2005 – 2006).

In July 2005, Russia reported its first outbreaks; domestic flocks were affected in six regions of western Siberia and dead wild birds were reported in the vicinities of some of these outbreaks (WHO, Global Alert Response, 2005). Kazakhstan reported its first outbreak in August 2005 in domestic birds (WHO, Global Alert Response, 2005). In the same month, 89 wild birds described as migratory species were reported infected at two lakes in Mongolia (WHO Report, 2007). Europe reported its first outbreaks of AI in October 2005 when infection was detected in domestic birds in Romania and Turkey (OIE, 2005). In the same month, Romania reported sporadic cases in wild birds as did Croatia and parts of Russia. In November, the virus spread to domestic birds in the Ukraine, and the Middle East reported its first case: a flamingo kept as a captive bird in Kuwait. During December, two outbreaks were reported in Russia in wild swans (species unreported) in regions near the Caspian Sea (Chris J.F, 2005). In the first half of 2006, the spread of HPAI H5N1 continued across Europe (Sabirovic *et al.*, 2006; Hesterberg *et al.*, 2007) and the Middle East and into Africa. Between January and May, infection was reported in 24 European countries with the majority of cases occurring in February and March in wild birds. During the same period, outbreaks were reported across central Asia and the Middle East, affecting domestic birds in Azerbaijan, India, Bangladesh, Pakistan, Iran and Iraq, with Azerbaijan also reporting infected wild birds. The first reported outbreak in Africa occurred in January (2006) in poultry in Nigeria, and by the end of April, eight other African nations had reported outbreaks: Burkina Faso, Cameroon, Djibouti, Egypt, Ghana, La Cote d'Ivoire, Niger and Sudan (OIE, 2008). By May 2006, reports of outbreaks in Europe, the Middle East and Africa had for the most part decreased in

frequency. Small numbers of cases of infection were reported in Hungary, Spain and the Ukraine in June; Pakistan and Russia in July; and one case was identified in a captive swan in Germany in August. Egypt was exceptional, continuously reporting outbreaks throughout 2006. It is also considered likely that outbreaks continued in poultry in Nigeria (UN System Influenza Coordinator & World Bank, 2007). Throughout the time HPAI H5N1 was spreading across central Asia, Europe, the Middle East and Africa; it maintained a stronghold in poultry in Southeast Asia. In 2006, outbreaks were reported in Cambodia, China, Hong Kong, Indonesia, Korea, Laos, Malaysia, Myanmar, Thailand and Vietnam (*OIE, 2008*).

Figure 4: Highly Pathogenic Avian Influenza H5N1outbreaks Outside Southeast Asia since 2005.



Source: WHO/OIE, 2007.

2.14 AVIAN INFLUENZA AND WETLANDS.

Given the ecology of the natural hosts of LPAI viruses, it is unsurprising that wetlands play a major role in the natural epidemiology of avian influenza. As with many other viruses, avian influenza virions survive longer in colder water (Stallknecht *et al.*, 1990; Lu *et al.*, 2003), and the virus is strongly suggested to survive over winter in frozen lakes in Arctic and sub-Arctic breeding areas. Thus, as well as the water bird hosts, these wetlands are probably permanent reservoirs of LPAI virus (Rogers *et al.*, 2004; Smith *et al.*, 2004) (re-)infecting water birds arriving from southerly areas to breed (shown in Siberia by Okazaki *et al.*, 2000 and Alaska by Ito *et al.*, 1995). Indeed, in some wetlands used as staging grounds by large numbers of migratory ducks, avian influenza viral particles can be readily isolated from lake water (Hinshaw *etal.*,1980).

An agricultural practice that provides ideal conditions for cross-infection and thus genetic change is used on some fish-farms in Asia: battery cages of poultry are placed directly over troughs in pig-pens, which in turn are positioned over fish farms. The poultry waste feeds the pigs, the pig waste is eaten by the fish or acts as a fertiliser for aquatic fish food, and the pond water is sometimes recycled as drinking water for the pigs and poultry (Greger, 2006). These kinds of agricultural practices afford avian influenza viruses, which are spread via the faecal-oral route, a perfect opportunity to cycle through a mammalian species, accumulating the mutations necessary to adapt to mammalian hosts. Thus, as the use of such practices increases, so does the likelihood that new influenza strains infectious to and transmissible between humans will emerge (Culliton, 1990; Greger, 2006).

As well as providing conditions for virus mutation and generation, agricultural practices, particularly those used on wetlands, can enhance the ability of a virus to spread. The role of Asian domestic ducks in the epidemiology of HPAI H5N1 has been closely researched and found to be central not only to the genesis of the virus (Hulse-Post *et al.*, 2005; Sims, 2007), but also to its spread and the maintenance of infection in several Asian countries (Shortridge and Melville, 2006). Typically this has involved flocks of domestic ducks used for 'cleaning' rice paddies of waste grain and various pests, during which they can potentially have contact with wild ducks using the same wetlands. Detailed research (Gilbert *et al.*, 2006; Songserm *et al.*, 2006) in Thailand has demonstrated a strong association between the HPAI H5N1 virus and abundance of free-grazing ducks. In Ghana, particularly in Sunyani, ducks are preferably reared in free-range or free-grazing. Gilbert *et al.* (2006) concluded that in Thailand "wetlands used for double-crop rice production, where free-grazing duck feed year round in rice paddies, appear to be a critical factor in HPAI persistence and spread".

2.15 WILDLIFE CONSERVATION IMPLICATIONS

Prior to HPAI H5N1, reports of HPAI in wild birds were very rare. The broad geographical scale and extent of the disease in wild birds is both extraordinary and unprecedented, and the conservation impacts of HPAI H5N1 have been significant. It is estimated that between 5-10% of the world population of Bar-headed Goose Anser *indicus* died at Lake Qinghai, China in spring 2005 (Chen *et al.*, 2005; Liu *et al.*, 2005). At least two globally threatened species have been affected: Black-necked Crane Grus *nigricollis* in China and Red-breasted Goose Branta *ruficollis* in Greece. Approximately 90% of the world population of Red-breasted Goose is confined to just

five roost sites in Romania and Bulgaria, countries that have both reported outbreaks, as also have Russia and Ukraine where they also over-winter (Birdlife International, 2007).However, the total number of wild birds known to have been affected has been small in contrast to the number of domestic birds affected, and many more wild birds die of other diseases each year. Perhaps a greater threat than direct mortality has been the development of public fear about water birds resulting in misguided attempts to control the disease by disturbing or destroying wild birds and their habitats. Such responses are often encouraged by exaggerated or misleading messages in the media. Currently, wildlife health problems are being created or exacerbated by unsustainable activities such as habitat loss or degradation, which facilitates closer contact between domestic and wild animals. Many advocate that to reduce risk of avian influenza and other bird diseases, there is a need to move to markedly more sustainable systems of agriculture with significantly lower intensity systems of poultry production. These need to be more biosecure, separated from wild water birds and their natural wetland habitats resulting in far fewer opportunities for viral cross-infection and thus pathogenetic amplification (Greger, 2006). There are major animal and human health consequences (in terms of the impact on economies, food security and potential implications of a human influenza pandemic) of not strategically addressing these issues. However, to deliver such an objective in a world with an ever-growing human population and with issues of food-security in many developing countries, will be a major policy challenge.

2.16 SITUATION OF HPAI H5N1 IN AFRICA

In Africa, HPAI H5N1 was reported in domestic birds in Nigeria, Egypt, Togo, Ghana and Benin; and is considered to have become endemic in Egypt (OIE, 2008; UN System Influenza Coordinator & World Bank, 2008). Also, the African Union/Interafrican Bureau for Animal Resources reported in February, 2009, that eleven African countries namely; Benin, Burkina Faso, Cameroon, Cote d'Ivoire, Ghana, Niger, Nigeria, Togo, Egypt, Djibouti and Sudan had been infected since the emergence of AI virus in domestic Africa. So far, Egypt has reported 144 human cases of H5N1 with 48 fatalities (Global Alert Response, WHO, June 2011). From March, 2009 to June, 2011, only Egypt reported outbreaks in domestic poultry. Nigeria also reported a single human case and fatality in January, 2007.

2.17 HIGHLY PATHOGENIC AVIAN INFLUENZA H5N1 IN GHANA

On April 24th, 2007, Ghana reported her first outbreak of HPAI H5N1 virus infection on a small-scale poultry farm situated at Kakasunanka near Michelle camp in the Tema Metropolitan area of the Greater Accra region. This virus was initially detected by a Rapid Test kit for Avian Influenza Virus Type A Antigen (Flu DETECT, Synbiotics Corporation) employed by the Emergency Preparedness Team of Veterinary Services Directorate (VSD). Further tests conducted at Noguchi Memorial Institute for Medical Research (NMIMR) and the OIE International Reference Laboratory, Padova, Italy and the United States NAMRU-3 in Cairo, Egypt, confirmed the presence of H5N1 virus in Ghana. An active search for the HPAI virus was conducted in the Tema Metropolis and three other infected farms at Adjei Kojo were found. A ban on movement and sale of poultry and poultry products in the Tema

Metropolis area was placed. A total of 13,391 birds were affected. About 36,400 birds were destroyed on the infected farms. On May 15, 2007, a second outbreak of HPAI H5N1 virus was detected on a backyard farm at New Dormaa in the Sunyani Metropolis of the Brong Ahafo region. The third outbreak was reported at Aflao in the Ketu South district of the Volta region on the June 13, 2007. In none of these outbreaks was there a human involvement.

The source of introduction of HPAI H5N1 virus into Ghana has not been traced. However, according to Mabbett (2007), the virus strain in Ghana was between 98.8% and 99.6% similar to other isolates from Burkina Faso, Cote d'Ivoire, Nigeria and Sudan.

2.18 EPIDEMIOLOGY OF AI VIRUS IN *BIRDS*

Wild aquatic birds, notably members of the orders *Anseriformes* (ducks and geese) and *Charadriiformes* (gulls and shorebirds), are carriers of the full variety of influenza virus A subtypes, and thus, most probably constitute the natural reservoir of all influenza A viruses (Webster 1992, Fouchier 2003, Krauss 2004, Widjaja 2004). While all bird species are thought to be susceptible, some domestic poultry species - chickens, turkey, guinea fowl, quail and pheasants - are known to be especially vulnerable to the sequelae of infection.

Avian influenza A viruses generally do not cause disease in their natural hosts. Instead, the viruses remain in an evolutionary stasis, as molecularly signalled by low *N/S* (non-synonymous vs. synonymous) mutation ratios indicating purifying evolution (Gorman 1992, Taubenberger 2005). Host and virus seem to exist in a state of a meticulously balanced mutual tolerance, clinically demonstrated by absence of disease

and efficient viral replication. Large quantities of virus of up to $10^{8.7}$ x 50% egg-infective dose (EID_{50}) per gram faeces can be excreted (Webster 1978). When transmitted to highly vulnerable poultry species, usually mild, if any, symptoms ensue. Viruses of this phenotype are referred to as low pathogenic (LPAIV) and, in general, only cause a slight and transient decline in egg production in layers or some reduction in weight gain in fattening poultry (Capua and Mutinelli 2001). However, strains of the subtypes H5 and H7 carry the potential to mutate to a highly pathogenic form after transmission and adaptation to the new poultry hosts. The highly pathogenic forms of H5 and H7 or of other subtypes had not been previously observed in wild birds (Webster 1998). Therefore, one may conclude that highly pathogenic forms are artificial, made possible as a result of man-made interference with a naturally balanced system.

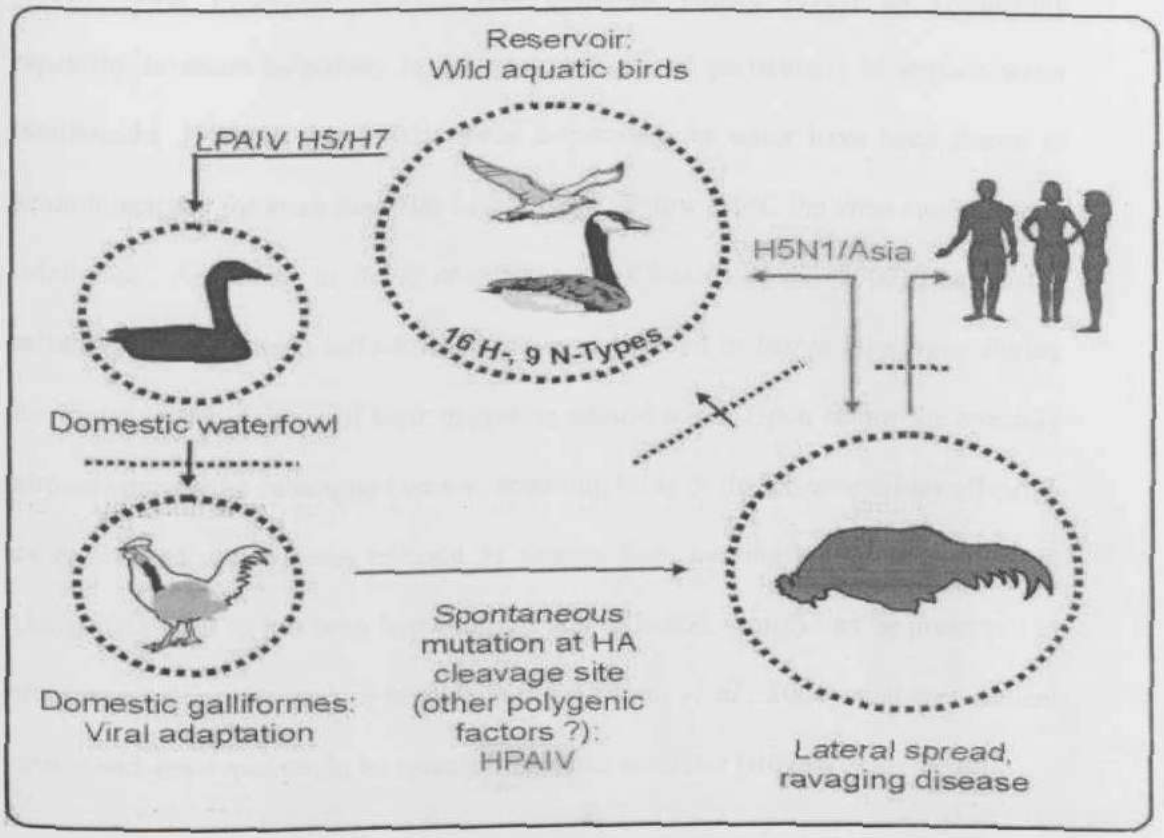
Once Highly Pathogenic Avian Influenza Virus (HPAIV) become established in domestic poultry, a highly contagious disease results and wild birds are no longer an essential factor for the spread (Swayne and Suarez 2000). This might have changed fundamentally since early 2005, when a large outbreak of the Asian lineage H5N1-related HPAI was observed among thousands of wild aquatic birds in a nature reservation at Lake Qinghai in the North West of China (Chen 2005, Liu 2005). As a result of this, further spread of this virus towards Europe during 2005 may have been initiated (OIE 2005).

Infected birds excrete virus in high concentration in their faeces and also in nasal and ocular discharges. Once introduced into a flock, the virus is spread from flock to flock through direct contact or by usual methods involving the movements of infected live birds or illegal trade or their unprocessed products, contaminated equipment, egg flats

feed trucks. Unintended mechanical passing-on of virus through human movements probably have been the main factors in the spread of HPAIV (Stegaman et al 2003).

Airborne transmission may occur if birds are in close proximity and with appropriate air movement. Birds are readily infected via instillation of virus into the conjunctival sac, or the tracheal. Preliminary field and laboratory evidence shows that Avian Influenza virus can be recovered from the yolk and albumen of eggs laid by hens at the peak of the disease. The possibility of vertical transmission is unresolved, however, it is unlikely that infected embryos could survive and hatch. Attempts to hatch eggs in disease cabinets from a broiler breeder flock at the height of disease failed to result in any Avian Influenza-infected chickens. However, broken contaminated eggs could be the source of virus infection to chicks after they hatch in the same incubator (Hoffmann 2000).

Fig 5: Diagram showing Avian Influenza Pathogenesis and Epidemiology



*LPAIV - low pathogenic avian influenza virus; HPAIV - highly pathogenic avian influenza virus; *HA - haemagglutinin protein;* dotted lines with arrows represent species barriers.

Source: Hoffmann, 2000.

2.19 TRANSMISSION BETWEEN BIRDS

Avian influenza viruses of low pathogenicity are genetically stable in wild water fowl (Webster *et al., 1992*). The infection cycle among birds depends on faecal-oral transmission chains. Apart from being directly transmitted from host to host, indirect spread via virus-contaminated water and fomites is an important route in contrast to influenza virus infections in mammals (humans, swine, and horses) where transmission by aerosols prevails. In birds, peak excretion titres of up to $10^{8.7}$ x 50 %

egg-infective dose (EID_{50}) per gram faeces have been measured with average titres grossly lower (Webster, 1978). Avian influenza viruses reveal an astonishing capability to retain infectivity in the environment and particularly in surface water (Stallknecht, 1990a+b, Lu, 2003). Virus suspensions in water have been shown to retain infectivity for more than 100 days at 17°C. Below -50°C the virus can be stored indefinitely. According to Ito *et al.* (1995) and Okazaki *et al.* (2000), that in the palearctic regions, avian influenza viruses are preserved in frozen lake water during the winter in the absence of their migrating natural hosts. Upon return for breeding purposes during the subsequent season, returning birds or their (susceptible) offspring are re-infected with viruses released by chance from melting environmental water. Along these lines, it has been hypothesized that influenza viruses can be preserved in environmental ice for prolonged time periods (Smith *et al.*, 2004), and that ancient viruses and genotypes might be recycled from this reservoir (Rogers *et al.*, 2004).

The introduction of H5 or H7 subtypes of LPAI viruses to susceptible poultry flocks is the basis of a chain of infection events which may lead to the *de novo* development of highly pathogenic biotypes. The risk that infection will be transmitted from wild birds to domestic poultry is greatest where domestic birds roam freely, share a water supply with wild birds, or use a water or food supply that might become contaminated by droppings from infected wild bird carriers (Capua, 2003; Henzler, 2003). Birds are infected by direct contact with virus-excreting animals and their excretions or through contact with (abiotic) vectors which are contaminated with virus-containing material. Once introduced into domestic flocks, LPAI virus may depend on a phase of adaptation to poultry species before excretion in amounts large enough to ensure sustained horizontal transmission within and between flocks. HPAI virus, once it has

arisen from an LPAI virus infected flock, spreads by similar means. So-called 'wet' markets, where live birds are sold under crowded conditions, are multiplicators of spread (Shortridge, 1998; Bulaga, 2003).

Biosecurity measures and the isolation of large poultry holdings, effectively prevent transmission from farm to farm by mechanical means, such as by contaminated equipment, vehicles, feed, cages, or clothing - especially shoes. An analysis of the Italian HPAI outbreaks in 1999/2000 revealed the following risks for transmission: movements of infected flocks (1.0 %), mediated contacts during transport of poultry to slaughter houses (8.5 %), neighbourhood within a one kilometer radius around infected premises (26.2 %), lorries used for transport of feed, bedding or carcasses (21.3 %), other indirect contacts through exchange of farm staff, working machines, etc. (9.4 %) (Marangon and Capua, 2005). There were no hints at aerogenic spread obtained during the Italian epizootic. However, during outbreaks in the Netherlands (2003) and Canada (2004), airborne spread has been considered (Landman and Schrier, 2004; Lees, 2004).

Until the emergence of the Asian lineage H5N1 HPAIV, a re-introduction of HPAIV from poultry into the wild bird population had not played any significant role. In April 2005, however, Asian lineage H5N1-associated disease surfaced at Lake Qinghai in North Western China affecting thousands of bar-headed geese and other migratory species of ducks, cormorants and gulls (Chen *et al.*, 2005; Liu, 2005).

Since late 2003, some H5N1 viruses have been encountered in Asia which were highly pathogenic for chickens but not for ducks (Sturm-Ramirez, 2005). Experimental infections using these isolates revealed a heterogeneous mixture with

respect to genetic analysis and plaque formation capacities in cell culture (Hulse Post, 2005). Ducks that survived infection with these isolates were shown to shed a virus population on day 17 that had lost its pathogenic potential for ducks. When clinical signs are used to screen for the presence of HPAIV H5N1 in the field, ducks may become the 'Trojan horse' of this virus (Webster, 2006).

2.20 TRANSMISSION TO HUMANS

Transmission of avian influenza viruses to humans, leading to the development of clinically overt disease is a rare event. Given the potential exposure of millions of people to HPAIV H5N1 in South East Asia, the actual number of documented human cases, although steadily growing over the past years, must still be considered as being comparatively low (http://www.who.int/csr/disease/avian_influenza/country/en).

The first association of the Asian lineage HPAIV H5N1 with respiratory illness in human beings was observed in Hong Kong in 1997, when six out of 18 H5N1 infected human cases died. These cases were epidemiologically linked to an outbreak of highly pathogenic H5N1 in live-bird markets (Claas, 1998; Yuen, 1998; Katz, 1999). The risk of direct transmission of the H5N1 virus from birds to humans seems to be greatest in persons who have close contact with live infected poultry, or surfaces and objects heavily contaminated with their droppings. Exposure risk is considered substantial during slaughter, defeathering, butchering and preparation of poultry for cooking (http://www.who.int/csr/don/2005_08_18/en/). The Asian lineage HPAI H5N1 virus can be found in all tissues - including the meat - throughout the bird's carcass. In several such instances, it was reported that the person who slaughtered or prepared a sick bird for consumption developed fatal illness, while family members

who participated in the meal did not (http://www.who.int/csr/don/2005_10_13/en/index.html). A H9N2 strain caused mild, influenza-like symptoms in two children in Hong Kong SAR in 1999 and in one child in mid-December 2003 (Saito, 2001; Butt, 2005). The H9N2 strain circulating in poultry at these times provoked significant symptoms and lethality rates in highly vulnerable species such as turkeys and chickens.

To date, there is no evidence that properly cooked poultry meat or poultry products are a source of human infection by the Asian lineage H5N1. As a general rule, the WHO recommends that meat be thoroughly cooked, so that all parts of the meat reach an internal temperature of 70°C. At this temperature, influenza viruses are inactivated, thus rendering safe any raw poultry meat contaminated with the H5N1 virus (WHO, 2005)

In Africa, Egypt as at June 2011 reported 144 human cases with 48 mortalities (Global Alert Response, WHO, June 2011). Nigeria recorded a single case of H5N1 in a 22 years lady who eventually died on January 17, 2007. Also, Ghana experienced its share of H5N1 outbreaks in birds in three different regions in 2007 without spillover to the human population.

Table 1: Cumulative Number of Confirmed Human Cases of Avian Influenza A (H5N1) Reported to WHO, June 2011.

Country	2003		2004		2005		2006		2007		2008		2009		2010		2011		Total	
	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D	C	D
Azerbaijan	0	0	0	0	0	0	8	5	0	0	0	0	0	0	0	0	0	0	8	5
Bangladesh	0	0	0	0	0	0	0	0	0	0	1	0	0	0	0	0	2	0	3	0
Cambodia	0	0	0	0	4	4	2	2	1	1	1	0	1	0	1	1	6	6	16	14
China	1	1	0	0	8	5	13	8	5	3	4	4	7	4	2	1	0	0	40	26
Djibouti	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	0	0	1	0
Egypt	0	0	0	0	0	0	18	10	25	9	8	4	39	4	29	13	25	8	144	48
Indonesia	0	0	0	0	20	13	55	45	42	37	24	20	21	19	9	7	7	5	178	148
Iraq	0	0	0	0	0	0	3	2	0	0	0	0	0	0	0	0	0	0	3	2
Laos	0	0	0	0	0	0	0	0	2	2	0	0	0	0	0	0	0	0	2	2
Myanmar	0	0	0	0	0	0	0	0	1	0	0	0	0	0	0	0	0	0	1	0
Nigeria	0	0	0	0	0	0	0	0	1	1	1	0	0	0	0	0	0	0	1	1
Pakistan	0	0	0	0	0	0	0	3	1	0	0	0	0	0	0	0	0	0	3	1
Thailand	0	0	17	12	5	2	3	3	0	0	0	0	0	0	0	0	0	0	25	17
Turkey	0	0	0	0	0	0	12	4	0	0	0	0	0	0	0	0	0	0	12	4
Vietnam	3	3	29	20	19	19	0	0	8	5	6	5	5	5	7	2	0	0	119	59
Total	*4*	*4*	*46*	*32*	*98*	*43*	*115*	*79*	*88*	*59*	*44*	*33*	*73*	*32*	*48*	*24*	*40*	*19*	*556*	*325*

Sources: Global Alert and Response (GAR), June, 2011. **C = Cases; D = Deaths.**

2.21 CONTROL MEASURES AGAINST HPAI

Due to its potentially devastating economic impact, HPAI is subject world-wide to vigilant supervision and strict legislation (Pearson, 2003; OIE Terrestrial Animal Health Code, 2005). Measures to be taken against HPAI depend on the epidemiological situation of the region affected. In the European Union (EU) where HPAIV is not endemic, prophylactic vaccination against avian influenza is generally forbidden. Thus, outbreaks of HPAI in poultry are expected to be conspicuous due to the clinically devastating course of the disease. Consequently, when facing such an outbreak, aggressive control measures, e.g. stamping out affected and contact holdings, are put in place, aiming at the immediate eradication of HPAI viruses and containing the outbreak at the index holding.

For these purposes, control and surveillance zones are erected around the index case with diameters varying from nation to nation (3 and 10 kilometers, respectively, in the EU). The quarantining of infected and contact farms, rapid culling of all infected or exposed birds, and proper disposal of carcasses, are standard control measures to prevent lateral spread to other farms (OIE - Terrestrial Animal Health Code). It is pivotal that movements of live poultry and also, possibly, poultry products, both within and between countries, are restricted during outbreaks.

In addition, control of H5 and H7 subtypes of LPAI in poultry, by testing and culling of acutely infected holdings, may be advisable in non-endemic areas in order to reduce the risk of a *de novo* development of HPAI virus from such holdings.

Specific problems of this eradication concept may arise in areas (i) with a high density of poultry populations (Marangon, 2004; Stegemann, 2004 and Mannelli, 2005) and

(ii) where small backyard holdings of free roaming poultry prevail (Witt and Malone, 2005). Due to the close proximity of poultry holdings and intertwining structures of the industry, spread of the disease is faster than the eradication measures. Therefore, during the Italian outbreak of 1999/2000 not only infected or contact holdings were destroyed, but also flocks with a risk of infection within a radius of one kilometer from the infected farm were pre-emptively killed. Nevertheless, eradication required four months and demanded the death of 13 millions birds (Capua, 2003). The creation of buffer zones of one to several kilometers around infected farms completely devoid of any poultry was also behind the successful eradication of HPAI virus in the Netherlands in 2003 and in Canada in 2004. So, not only the disease itself, but also the pre-emptive culling of animals led to losses of 30 and 19 million birds, respectively. In 1997, the Hong Kong authorities culled the entire poultry population within three days (on the 29th, 30th, and 31st December: 1.5 million birds). The application of such measures, aimed at the immediate eradication of HPAI virus at the cost of culling also non-infected animals, may be feasible on commercial farms and in urban settings. However, this will afflict the poultry industry significantly and also prompts ethical concern from the public against the culling of millions of healthy and uninfected animals in the buffer zones.

Such measures are most difficult to implement in rural areas with traditional forms of poultry holdings where chickens and ducks roam freely and mingle with wild birds or share water sources with them. Moreover, domestic ducks attract wild ducks and provide a significant link in the chain of transmission between wild birds and domestic flocks (WHO, 2005). These circumstances may provide the grounds for HPAI viruses to gain an endemic status.

Endemicity of HPAI in a certain region imposes a constant pressure on poultry holdings. As restrictions can not be upheld over prolonged periods without vital damage to a country's poultry industry or, in the developing world, leading to a serious shortage of protein supply for the population, other measures must be considered.

Vaccination has been widely used in these circumstances and may also be a supplementary tool in the eradication process of outbreaks in non-endemic areas.

2.22 BIOSECURITY MEASURES

The aim of a bio-security programme is to reduce sources and causes of contamination to enable the supply of a healthy, safe and reliable product (Artois *et al.*, 2009). The Department for Environment, Food and Rural Affairs (Defra) 2007 guidelines and the Institute de Selection Animale (ISA) of Netherlands outlines the following bio-security programme for effective control of poultry diseases. These include a buffer zone and clean area. Persons entering clean area should at least change shoes and cloths. Also, equipments entering the clean area should be cleaned and disinfected. Again, all materials entering this zone should be stored for at least two days in a clean, dry and rodents freed room before.

Visitors entering poultry premises should fully understand proper hygienic procedures and records must be kept on all visitors. Farms workers must wash their hands thoroughly with soap after any farm activity. Also, care should be taken at all times to protect the health and safety of farm workers and visitors (OIE Guidelines).

Poultry houses must be cleaned and disinfected after emptying them. Footbaths should be replenished with additional disinfectant daily to maintain sufficient depth and the whole contents of the bath renewed once soiled or at least twice a week (Defra, 2007).

2.23 VACCINATION

In the veterinary, vaccination pursues four goals: (i) protection from clinical disease, (ii) protection from infection with virulent virus, (iii) protection from virus excretion, and (iv) serological differentiation of infected from vaccinated animals (DIVA principle).

In the field of influenza vaccination, neither commercially available nor experimentally tested vaccines have been shown so far to fulfill all of these requirements (Lee and Suarez, 2005). The first aim, which is the protection from clinical disease induced by HPAIV, is achieved by most vaccines. The risk of infection of vaccines with and excretion of, virulent field virus is usually reduced but not fully prevented. This may cause a significant epidemiological problem in endemic areas where exhaustive vaccination is carried out: vaccinated birds which appear healthy may well be infected and excrete the field virus 'under cover' of the vaccine. The effectiveness of reduction of virus excretion is important for the main goal of control measures, that is, the eradication of virulent field virus. The effectiveness can be quantified by the replication factor r0. Assuming a vaccinated and infected flock passes on the infection on average to less than one other flock ($r0 < 1$), the virulent virus is, on mathematical grounds, prone to be extinguished (van der Goot, 2005). When dealing with vaccination against the potentially zoonotic H5N1 virus, reduction of virus excretion also reduces the risks of transmission to humans, since a significant

dose of virus seems to be required to penetrate the species barrier between birds and humans. Also, a DIVA technique allows the tracing of field virus infections by serological means in vaccinated birds.

Various vaccine concepts have been developed. Most are still based on inactivated, adjuvant whole virus vaccines which need to be applied by needle and syringe to each animal separately.

Inactivated homologous vaccines, based on the actual HPAI strain, induce proper protection but do not allow a distinction between vaccines and infected birds serologically. Since the vaccine is made from the current HPAI virus, there is an inherent delay before such vaccines can be used in the field.

Inactivated heterologous vaccines, in contrast, can be used as marker vaccines when the vaccine virus expresses the same HA- but a different NA-subtype compared to the field virus (e.g. H5N9 vaccine vs. H5N2 HPAI). By detection of NA subtype-specific antibodies, vaccines and infected birds can be distinguished (Cattoli, 2003). However, these methods can be laborious and may lack sensitivity. Nevertheless, such vaccines can be kept in vaccine banks comprising several H5- and H7-subtypes with discordant NA subtypes. Reverse genetics will greatly aid in producing vaccines both for veterinary and medical use with the desired HxNy combinations in a favourable genetic background (Liu, 2003; Neumann, 2003; Lee, 2004; Chen 2005 and Stech 2005). Currently, inactivated heterologous vaccines have been used the H5N1 hot spots of South East Asia as well as in Mexico, Pakistan and Northern Italy (Garcia, 1998 and Swayne 2001). As an alternative DIVA system for use with inactivated vaccines, the detection of NS-1 specific antibodies has been proposed (Tumpey,

2005). These antibodies are generated at high titres by naturally infected birds, but at considerably lower titres when inactivated vaccines are used.

Recombinant live vector-engineered vaccines express an H5 or H7 HA gene from the backbone of viruses or bacteria capable of infecting poultry species (e.g. fowl pox virus [Beard, 1991, Swayne, 1997+2000c], laryngotracheitis virus [Lueschow, 2001and Veits, 2003] or Newcastle Disease virus [Swayne, 2003] among others). Being live vaccines, mass application via water or sprays is often feasible. While allowing for a clear-cut DIVA distinction, a pre-existing immunity towards the vector virus, however, will grossly interfere with vaccination success. Some field experience with fowl pox recombinants has been collected in Mexico and the U.S. (Kamps *et al.*, 2006)

Finally, successful use of recombinant expressed HA proteins and of DNA vaccination using HA-expressing plasmids has been experimentally proven (Crawford 1999 and Kodihalli 1997).

Vaccination has been used on a nationwide scale in several countries in South East Asia (Normile, 2005).

2.24 CLINICAL PRESENTATION

Following an incubation period of usually a few days (but rarely up to 21 days), depending upon the characteristics of the virus strain, the dose of inoculum, the species, and age of the bird, the clinical presentation of avian influenza in birds is variable and symptoms are fairly unspecific (Elbers, 2005). Therefore, a diagnosis solely based on the clinical presentation is a challenge.

The symptoms following infection with low pathogenic (LP) AI virus may be as discrete as ruffled feathers, transient reductions in egg production or weight loss combined with a slight respiratory disease (Capua and Mutinelli, 2001). Some LP strains such as certain Asian H9N2 lineages, adapted to efficient replication in poultry, may cause more prominent signs and also significant mortality (Bano, 2003 and Li, 2005).

In its highly pathogenic form, AI illness in chickens and turkeys is characterized by a sudden onset of severe symptoms and a mortality that can approach 100 % within 48 hours (Swayne and Suarez, 2000). Spread within an affected flock depends on the form of rearing: in herds which are litter-reared and where direct contact and mixing of animals is possible, spread of the infection is faster than in caged holdings but would still require several days for complete contagion (Capua, 2000). Often, only a section of a farm is affected. Many birds die without premonitory signs so that sometimes poisoning is suspected in the beginning (Nakatami, 2005). It is worth noting, that a particular HPAI virus isolate may provoke severe disease in one avian species but not in another. In live poultry markets in Hong Kong prior to a complete depopulation in 1997, 20 % of the chickens but only 2.5 % of ducks and geese harboured H5N1 HPAIV while all other galliforme, passerine and psittacine species tested virus-negative and only the chickens actually showed clinical disease (Shortridge, 1998).

In industrialized poultry holdings, a sharp rise followed by a progressive decline in water and food consumption can signal the presence of a systemic disease in a flock. In laying flocks, a cessation of egg production is apparent. Individual birds affected by HPAI often reveal little more than severe apathy and immobility (Kwon, 2005).

Oedema, visible at feather-free parts of the head, cyanosis of comb, wattles and legs, greenish diarrhoea and laboured breathing may be inconsistently present. In layers, soft-shelled eggs are seen initially, but any laying activities cease rapidly with progression of the disease (Elbers, 2005). Nervous symptoms including tremor, unusual postures (torticollis), and problems with co-ordination (ataxia) dominate the picture in less vulnerable species such as ducks, geese, and ratites (Kwon, 2005). During an outbreak of HPAI in Saxonia, Germany, in 1979, geese compulsively swimming in narrow circles on a pond were among the first conspicuous signs leading to a preliminary suspicion of HPAI.

2.25 LABORATORY DIAGNOSIS OF AVIAN INFLUENZA DISEASE

2.25.1 Collection of Specimens

Specimens should be collected from several fresh carcasses and from diseased birds of a flock. Ideally, adequate sampling is statistically backed up and diagnosis is made on a flock basis. When sampling birds suspected of HPAI, safety standards must be observed to avoid exposure of the sample collectors to potentially zooanthroponotic HPAIV (Bridges, 2002). Guidelines have been proposed by the Center for Disease Control (CDC, 2005).

For virological assays, swabs obtained from the cloacae and the oropharynx generally allow for sound laboratory investigation. The material collected on the swabs should be mixed into 2-3 ml aliquots of a sterile isotonic transport medium containing antibiotic supplements.

2.25.2 Transport of Specimens

Swabs, tissues and blood should be transported chilled but not be allowed to freeze. If delays of greater than 48 hours are expected in transit, these specimens should be frozen and transported on dry ice. In all cases, transport safety regulations (e.g International Air Transport Agency rules) should be punctiliously observed to avoid spread of the disease and accidental exposure of personnel during transport. It is highly advisable to contact the assigned diagnostic laboratory before sending the samples and, ideally, even before collection.

2.25.3 Direct Detection of AI Virus Infections

Basically, there are two (parallel) lines of diagnostic measures that attempt to (i) isolate and subtype the virus by classical methods (OIE Manual, 2005) and (ii) molecular detection and characterization of the viral genome.

(i) Conventionally, AI virus is isolated by inoculation of swab fluids or tissue homogenates into 9- to 11-day-old embryonated chicken eggs, usually by the chorioallantoic sac route (Woolcock, 2001). Depending on the pathotype, the embryos may or may not die within a five-day observation period and usually there are no characteristic lesions to be seen in either the embryo or the allantois membrane (Mutinelli, 2003b). Eggs inoculated with HPAI virus containing material usually die within 48 hours. The presence of a haemagglutinating agent can be detected in harvested allantoic fluid using chicken red blood cells. Haemagglutination (HA) is an insensitive technique requiring at least 10^6 particles per ml. If only a low virus concentration is present in the inoculum, up to two further passages in embryonated eggs may be necessary for some LPAI viral strains, in order to produce enough virus

to be detected by HA. In the case of HPAI virus, a second passage using diluted inoculum may be advantageous for the optimal production of the haemagglutinating agent.

Haemagglutinating isolates are antigenically characterised by Haemagglutination inhibition (HI) tests using (mono-) specific antisera against the 16 H subtypes and, for control, against the different types of avian paramyxoviruses which also display haemagglutinating activities. The NA subtype can be subsequently determined by neuraminidase inhibition assays, again requiring subtype-specific sera (Aymard, 2003). In case isolates of the H5 or H7 lineages are encountered, their intravenous pathogenicity index (IVPI) needs to be determined to distinguish between LP and HP biotypes (Allan, 1977). This is achieved by intravenous (iv) inoculation of ten 6-week old chickens with the egg-grown virus isolate (0.1 ml of a 1 in 10 dilution of allantoic fluid containing a HA titre greater than 1 in 16). The chickens are observed over a period of ten days for clinical symptoms. Results are integrated into an index which indicates a HPAI virus when values greater than 1.2 are obtained. Alternatively, a HPAI isolate is encountered when at least seven out of ten (75 %) inoculated chickens die within the observation period.

The application of classical procedures can lead to a diagnosis of HPAI within five days but may demand more than a fortnight to rule out the presence of AIV. In addition, high quality diagnostic tools (Specific Pathogen-Free eggs, H- and N-subtype specific antisera) and skilled personnel are a prerequisite. Currently, there are no cell culture applications for the isolation of AIV that can achieve the sensitivity of embryonated hen eggs (Seo, 2001).

(ii) A more rapid approach, especially when exclusion of infection is demanded, employs molecular techniques. The presence of influenza A specific RNA can be detected through the reverse transcription-polymerase chain reaction (RT-PCR) targeting fragments of the M gene, the most highly conserved genome segment of influenza viruses (Fouchier, 2000 and Spackman, 2002), or the nucleocapsid gene (Dybkaer, 2004). When a positive result is obtained, RT-PCRs amplifying fragments of the haemagglutinin gene of subtypes H5 and H7 are run to detect the presence of notifiable AIVs (Dybkaer, 2004 and Spackman, 2002). A molecular diagnosis of the pathotype (LP versus HP) is also feasible after sequencing a fragment of the HA gene spanning the endoproteolytic cleavage site. Isolates presenting with multiple basic amino acids are classified as HPAI. PCRs and other DNA techniques have been described for the detection of Asian lineage H5N1 strains (Collins, 2002 and Payungporn, 2004). Non-H5/H7 subtypes can be identified by a canonical RT-PCR and subsequent sequence analysis of the HA-2 subunit (Phipps, 2004). There are also specific primers for each NA subtype; a full characterisation might be achievable within three days, especially when real time PCR techniques are used (Perdue, 2003; Lee and Suarez, 2004). Furthermore, DNA chips have been developed which should further streamline the typing of AI viruses (Li, 2001 and Kessler, 2005). An exclusion diagnosis is thus possible within a single working day.

The disadvantages of molecular diagnostics are the cost of equipment and consumables, which if available, will allow analyses of many samples by fewer personnel in grossly shorter times in comparison to virus isolation in eggs. However, it should be noted that for each PCR or hybridization reaction, in contrast to virus isolation in eggs, there is an intrinsic uncertainty related to the presence of specific

mutations in a given isolate at the binding sites of primers and/or probes which might render the assay false negative.

Thus, a combination of molecular (e.g. for screening purposes) and classical methods (e.g. for final characterisation of isolates and confirmation of diagnosis of an index case) may help to counterbalance the disadvantages of the two principles.

Rapid assays have been designed for the detection of viral antigen in tissue impression smears and cryostat sections by use of immunofluorescence or by antigen-capture enzyme-linked immunosorbent assay (ELISA) and dip-stick lateral flow systems in swab fluids. These techniques have been less sensitive than either virus isolation or PCR, and therefore might be difficult to approve for a legally binding diagnosis, especially of an index case (Selleck, 2003 and Cattoli, 2004).

2.25.3a Real-Time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR).

Principles of rRT-PCR

The Polymerase Chain Reaction (Freymuth *et al.*, 1995) has been used as the new gold standard for detecting a wide variety of templates across a range of scientific specialties including virology. The method utilizes a pair of synthetic oligonucleotides or primers, each hybridizing to one strand of a dsDNA, target, with the pair spanning a region of interest which will be reproduced exponentially. The hybridized primer acts as a substrate for a DNA polymerase which creates a complimentary strand through addition of deoxynucleoside. The process can be summarized in 3 steps: dsDNA separation at temperatures >90°C, primer annealing at 50 – 70°C and extension at 72 –

78°C. The main problem with this conventional PCR is evaluating generated amplicons (Guatelli *et al.*, 1989).

In contrast to conventional PCR in a real time PCR assay, amplicons can be visualized as the amplification progresses. Real Time PCR is described as 'kinetic' PCR. The monitoring of accumulating amplicon is made possible by the labeling of primers, probes or amplicons with flurogenic molecules. There are 5 chemistries available for real-time PCR. They include: DNA-binding fluorophores, Linear oligoprobes, 5' Nuclease oligoprobes, Hairpin oligoprobes and Self-fluorescing amplicons. For this study, the chemistry used was the 5' Nuclease oligoprobes where in addition to a forward and reverse primer the reaction also involved a fluorogenic labeled oligoprobe. This probe was labeled at the 5' end with a reporter fluorophore (6-carboxy- fluorescein, FAM) and at the 3' end with a quencher fluorophore (6-carboxy-tetramethyl – rhodamine, TAMRA). When the oligoprobe hybridizes to its template, the fluorophores were released due to hydrolysis of the oligoprobe component of the probe and target duplex by the enzyme *Taq* via its 5' exonuclease activity. A combined fluorescence excitation and detection platform which is also a thermal cycler is used to detect fluorescence activity. This platform is a real-time PCR machine. During the detection, an amplification curve which is a typical sigmoid curve is obtained from a plot of fluorescence activity against the cycle number. Early amplification cannot be viewed because the detection signal is indistinguishable from the background. The point at which the fluorescence passes from insignificant levels to clearly distinguishable levels is called the threshold cycle C_T. The C_T value is proportional to the number of target copies present in the sample. For this study a C_T value < 30 was considered as positive for the target template (RNA).

2.25.4 Indirect Detection of AIV Infections

Serology on a herd basis may be useful for screening purposes (Beck, 2003). For the detection of AIV-specific antibodies in serum samples from birds, or in egg yolk in the case of laying flocks, the Haemagglutination inhibition (HI) assay using reference subtype antigens still represents the gold standard. Group-specific antibodies (influenza virus type A) against the nucleocapsid protein can also be detected by agar gel immunoprecipitation and by enzyme-linked immunosorbent assays (ELISA) (Snyder *et al.*, 1985; Meulemans *et al.*, 1987 and Jin *et al.*, 2004). Competitive ELISA formats allow the examination of sera of all bird species, independent from the availability of species-specific conjugates (Shafer *et al.*, 1998 and Zhou, 1998). An ELISA format for the detection of H7-specific antibodies has been reported (Sala *et al.*, 2003).

Subtype-specific antibody kinetics depend on the viral strain characteristics and, primarily, on the host species. In gallinaceous birds, AIV-specific antibodies reliably become detectable during the second week following exposure; antibodies in egg yolk are detectable after a delay of a few days (Beck, 2003). The production and detection of antibodies in *Anatidae* species are much more variable (Suarez and Shultz-Cherry, 2000).

2.25.5 Pathology of AI

Birds that die of peracute disease may show minimal gross lesions, consisting of dehydration and congestion of viscera and muscles

In birds that die after a prolonged clinical course, petechial and ecchymotic haemorrhages occur throughout the body, particularly in the larynx, trachea, proventriculus and epicardial fat, and on serosal surfaces adjacent to the sternum. There is extensive subcutaneous oedema, particularly around the head and hocks. The carcass may be dehydrated. Yellow or grey necrotic foci may be present in the spleen, liver, kidneys and lungs. The air sac may contain an exudate. The spleen may be enlarged and haemorrhagic (Parkins and Swayne, 2003)

Avian influenza is characterised histologically by vascular disturbances leading to oedema, haemorrhages and perivascular cuffing, especially in the myocardium, spleen, lungs, brain and wattles. Necrotic foci are present in the lungs, liver and kidneys. Gliosis, vascular proliferation and neuronal degeneration may be present in the brain (Parkins and Swanyne 2003; Kwon *et al.*, 2005; Brojer *et al.*, 2009).

2.25.6 Differential Diagnosis of AI With other Diseases

clinically, the less severe forms of AI may be confused with many other respiratory or enteric diseases in poultry (Elbers, 2005). However, in the laboratory, AI can only be differentiated from other acute poultry respiratory diseases such as Newcastle, infectious laryngotracheitis, duck plague, acute fowl cholera and other septicemia diseases, by serological tests (Agar gel-diffusion test) or virus isolation and molecular detection by the RT-PCR (Animal health advisory leaflet 8; 1996). Avian Influenza should be suspected in any disease outbreak in poultry that persists despite the application of preventive and therapeutic measures for other diseases (Elbers, 2005).

Newcastle disease which has a very similar signs and lesions as AI is characterized by the sudden onset of watery discharge from the nostrils, labored breathing, facial

swelling, paralysis, trembling and twisting of the neck. Mortality ranges from 10 to 80% depending on host immunity. It causes also, drastic reduction in egg-laying and production of soft-shelled eggs. Lesions of Newcastle disease include hemorrhages of peyers patches in the intestines, hemorrhages in the ovaries, proventiculus, intestine lining and caecal tonsils.

2.26 ECONOMIC CONSEQUENCES OF HPAI

Outbreaks of highly pathogenic avian influenza can be catastrophic for single farmers and for the poultry industry of an affected region as a whole. Economical losses are usually only partly due to direct deaths of poultry from HPAI infection. Measures established to prevent further spread of the disease levy a heavy toll. Nutritional consequences can be equally devastating in developing countries where poultry is an important source of animal protein. Once outbreaks have become widespread, control is difficult to achieve and may take several years (WHO, 2004).

This is illustrated by the Southeast Asian outbreaks which resulted in high mortalities, resulting in the destruction of over one hundred and fifty million (150,000,000) birds accounting to lost in revenue estimated at over ten billion US dollars (Diouf 2005). This was a serious setback for the Agricultural development. There was associated poverty for many rural farmers who depended solely on small scale backyard poultry farming for household income.

CHAPTER THREE

METHOD

3.1. STUDY AREA

The study was conducted in the Sunyani Municipality, one of the twenty-two districts of the region which is the capital of the Brong Ahajo region. It lies between latitude 7^0 20/N and 7^0 05/N and longitude 2^0 30/W and 2^0 10/W and shares boundaries with Sunyani West District to the north, Dormaa District to the west, and Asutifi District to the South and Tano North District to the East. The Municipality has a total land area of 829.3 square kilometers (320.1 square miles). One third of the land area is not inhabited or cultivated. The Municipality falls within the wet semi-equatorial climatic zone of Ghana. The mean monthly temperatures vary between 23° C and 33 ° C, the lowest in August and the highest being observed around March and April. The relative humidity is high averaging between 75 and 80 percent during the rainy seasons, while it averages 70 percent during the dry seasons of the year.

In 2002, the population of Sunyani Municipality was 101,145. As at 2010, with a growth rate of 3.8 percent, the estimated population is 147,301. The population density of the Municipality is 122 persons per square kilometer (Municipal Planning and Coordinating Unit computation, 2010). The population in the Municipality is generally concentrated in the three largest localities (Sunyani, Abesim and New Dormaa) which hold about 74.3% of the population, with only 25.7% distributed among the other settlements. The male female ratio shows a ratio of 50.4 females to 49.6 males. There are nine main identifiable ethnic groups in the Municipality. Akan, the majority ethnic group constitutes 71.1%; Ga Dangbe presents 2.1% where as Ewe

constitutes 3.2%. In total, the northern tribes (Guan, Gurma, Mole-Dagbani, Grusi and Mande) in the Municipality constitute 19.3% (Ghana Statistical Service, Primary Health Care, 2000).

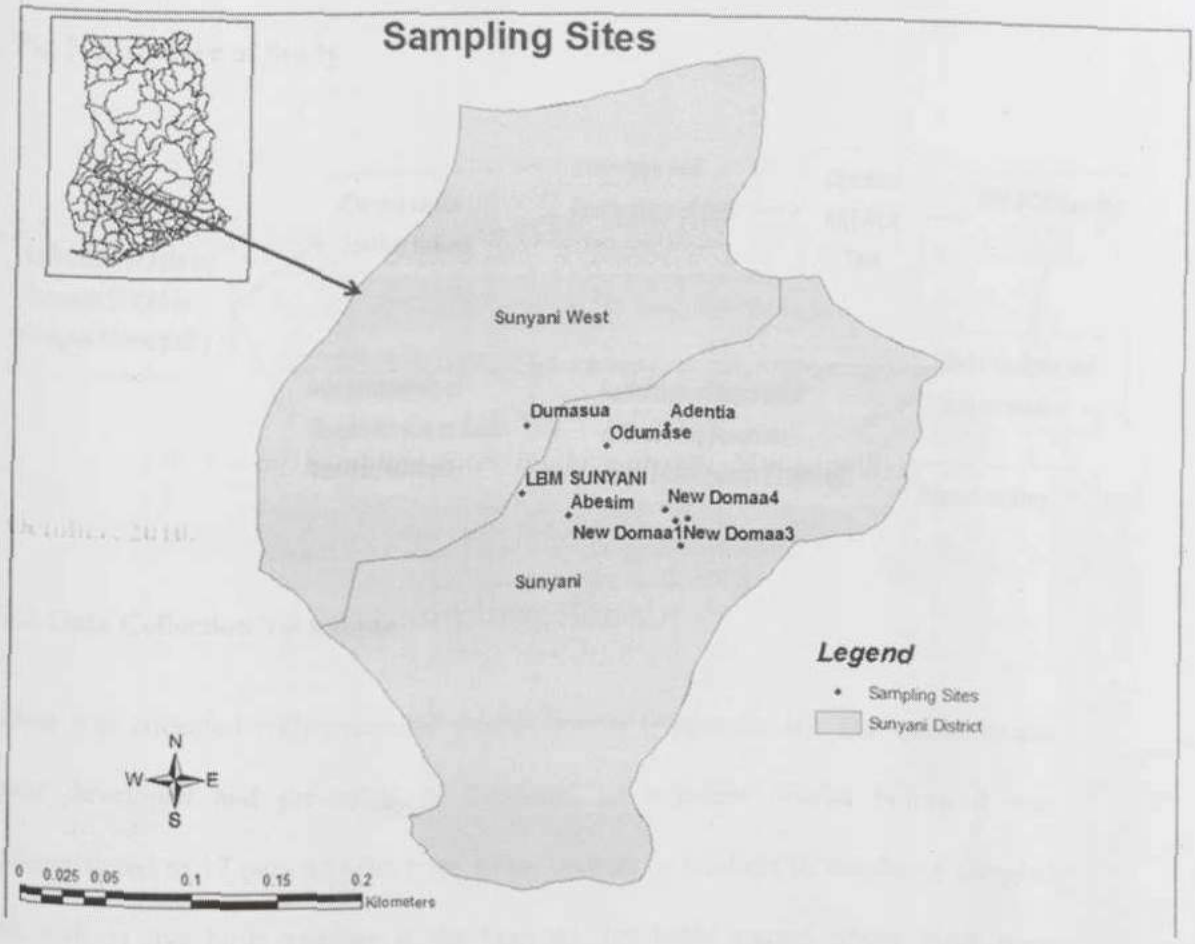
The people in the district are mostly Christians accounting for about 84%. Moslems population is about 10%, Traditionalists taking about 2% and others 4%. Despite the religion diversity, there is religious tolerance in the district.

The mainstay of the District economy is Agriculture which employs about 60% of the active labor force. Most of the district's households are engaged in agricultural related activities. Farming in the district is largely carried out on small-scale basis and the main food crops cultivated in commercial quantities in the district include maize, plantain, cassava, yam, cocoyam, tomatoes and pepper. The district is also known for the production of cocoa and coffee.

The poultry industry specifically table egg production operates on a large scale level in the region. Poultry production in the region is one of the largest in the nation. Livestock such as cattle, sheep, goats and grass cutters are also reared in the district.

The poultry population for the Brong Ahafo region was 2,503,559 with ducks accounting for only about 1% or 25,036 (MOFA, 2005).

Fig 6: Map showing Sampling Sites in the Sunyani Municipality, July 2009-October, 2010.



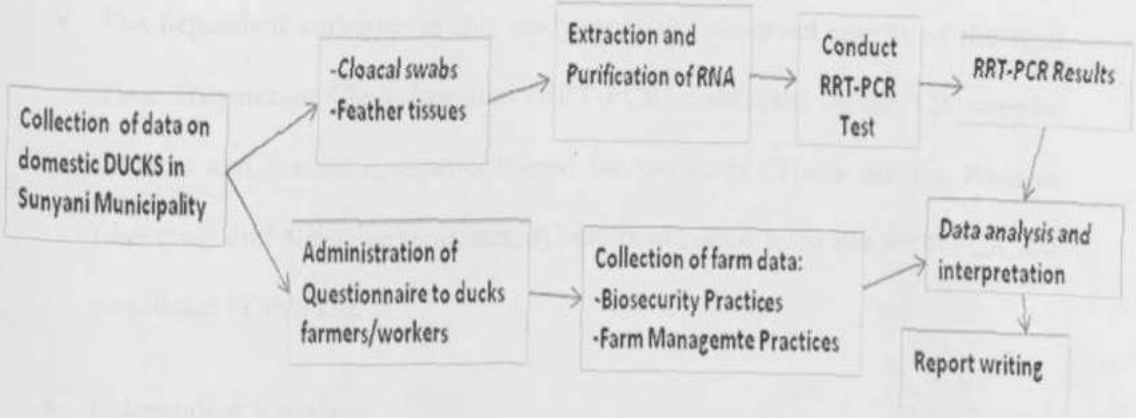
Source: *Epidemiology Unit, VSD, Accra Ghana, 2011.*

3.2 STUDY DESIGN

A descriptive cross-sectional study using active surveillance approach was carried out as indicated in the scheme below. It entailed simultaneous collection of cloacal swabs and feather tissues from three hundred eighty four domestic ducks, and the administration of a structured questionnaire to 17 ducks owners/workers on issues of

husbandry practices and bio-security at their farms or premises (households). The study was carried out from July 2009 to August 2010.

Fig 7: Structure of Study



3.3 Data Collection Technique

Data was collected with structured questionnaires (Appendix II). The questionnaire was developed and pre-tested in Berekum, an adjacent district before it was administered to 17 persons who were either owners or workers of the ducks sampled as well as live birds vendors at the Sunyani live birds market whose birds were sampled. Questionnaire administration was done simultaneously with sample collection.

3.4 Data Quality Control

Data collection was designed with systematic checks to avoid errors during questionnaire administration and data entry. Data collected was double entered into a computer on the same day and inconsistencies were immediately corrected. Three research assistants were trained to assist to administer the questionnaires under the guidance of a field supervisor. Forms filled incorrectly were rectified within a day.

3.5 VARIABLES

- Dependent Variables

✓ The dependent variables in this study were the observed results of the Real Time Polymerase Chain Reaction (RRT-PCR) conducted on the 526 samples (cloacal and feather tissues) collected for the study. These are the Positive (detected) and Negative (undetected) results obtained from the RRT-PCR test conducted (Table 13).

- Independent Variables

✓ These were variables that portrayed the farmers' biosecurity practices (either adherence or non-adherence) which could influence the outcome of the dependent variable(s). Examples of biosecurity practices observed in this study included;

i. Use of Personal Protective Equipments (PPEs) by farm workers/visitors, personnel disinfection etc.

ii. Farm hygiene (cleaning and disinfection of farm premises, use of footbaths, perimeter fencing, proper disposal of carcasses and farm waste etc.)

iii. Regulate human and vehicular movements to and from the farm, disinfection of vehicles entering farm premises etc.

Non-adherence to any or all of the above practices and others was considered a risk factor that could lead to the re-introduction and spread of AI virus and would influence the outcome of the dependent variable.

3.6 Study Population

Sunyani Municipality had an estimated ducks population of 25,036 (according to the Statistics, Research and Information Directorate data, MOFA, 2005) before the 2007 H5N1 outbreak. Many of these ducks were destroyed during the 2007 HPAI outbreaks. Presently, fourteen ducks farmers in the Municipality who abandoned the trade because of the outbreaks in 2007 have become operational again. The population of ducks keepers in the Municipality is estimated at 107. Duck population in the area was estimated at 5,000 after collecting census data from the various active ducks rearing sites in the Municipality.

3.7 SAMPLE SIZE DETERMINATION

The sample size was calculated by the Epi info version 3.4.1 at 95% confidence level, absolute precision of 5% and assuming 0.5 prevalence of Avian Influenza among domestic ducks in Sunyani.

$$N=z^2\, p\,(1-p)/d^2 = \frac{(1.96)^2(0.5)(0.5)}{(0.05)^2} = \frac{0.9604}{0.0025} = 384.16$$ **(Jones *et al.*, 2003)**

- where N= sample size,

- z= risk of Type 1 error=1.96 at 95% confidence level
- p= prevalence of *AI* = *0.5* (arbitrary proxy)
- d= absolute precision = 5% = 0.05
- Total number of domestic ducks sampled =**384**
- Sample size = 384 cloacal swabs plus 142 feather tissues = **526**

3.8 SAMPLING METHOD

Thirteen (13) epidemiological units (epi units) or communities in the Sunyani Municipality were identified for the study. Simple random sampling method was used to select a community at a time for ducks to be identified in households and farms within that community for sample collection. This procedure was repeated till the desired sample size for the study was attained from nine (9) sites in six communities.

At each site, the number of ducks selected for sample collection was determined by the population of ducks at the site (See Table 2). Eventually, when the number of ducks to be sampled on site was determined, we then used simple random sampling method (birds were numbered and balloted) to pick birds for sample collection. However, in the only commercial farm where the population of ducks was over thousand (>1,000) and it was not possible to mark birds individually for balloting, we conveniently picked them from different locations of the farm house (i.e confined ducks) for sampling.

Table 2: **Sampling of Ducks and Geese using 0.5 prevalence of AI Virus.**

Ducks and Geese Population/Epidemiological Unit	**Sample size**
<15	Sample all
20	19
25	24
30	23
35	27
45	40
50-75	45
80-100	47
110-150	47
160-200	50
200-700	55
≥800	59

Source: FAO/OIE HPAI Reference Laboratory, 2007

Table 1 shows a standard sampling formula for ducks and geese at 50% prevalence of avian influenza disease in a farm with absolute precision of 5%. Depending on the

size of the farm or number of ducks at a site, an X number of ducks or geese are selected for sample collection as indicated on the Table 2.

3.9 ETHICAL CONSIDERATIONS AND CONSENT

Approval for the study was duly obtained from the Scientific Technical Committee of Noguchi Memorial Institute for Medical Research (NMIMR). A written informed consent was obtained from the Brong Ahafo Regional Veterinary Officer, Commercial and Backyard ducks owners, and vendors at the Live Birds Market in the selected communities to carry out the study (Appendix I).

3.10 SAMPLE COLLECTION AND PROCESSING

Cloacal swabs and feather tissues (calamuses) were obtained from backyard ducks, a commercial farm and a live birds market. For each duck sampled, a cloacal swab and a feather calamus were to be collected. However, we were able to collect only one hundred and forty two (142) feather tissues as against three hundred and eighty four (384) cloacal swabs. This was due to the shortage of VTM.

All field samples collected were pooled separately by type (cloacal swabs separate from feather tissues). Two cloacal swabs from two (2) different birds from the same site were placed into a 2.0 ml tube while ten (10) feather calamuses with similar characteristics were pooled into a 25 ml vacutainer tube with both tubes containing 2

ml and 5 ml of viral transport medium (VTM) respectively. VTM was prepared in the Virology Department of NMIMR with 2.5% Veal Infusion Broth (SIGMA), 0.5% Bovine Serum Albumin (SIGMA), 100µg/ml Gentamicin Sulphate (SIGMA) and 2 µg/ml Fungizone (Amphotericin B) solution.

The vacutainer tubes containing these samples were properly labeled with information that described a unique identification number and dates of collection. They were placed on ice in "cold boxes" from the field and transported to the Sunyani Veterinary office where they were stored at -70 °C. At the end of the samples collection, they were finally transported on ice again in a "cold box" to the Virology Department of NMIMR in Accra the same day where they were kept at -70 °C until RNA extraction and eventually RRT-PCR was carried out.

In the laboratory, feather calamuses were minced into the transport medium and vortexed several times. The supernatant was harvested and stored at -70°C until analyses.

3.11 Preparation for RNA Extraction from Samples collected

The 526 samples (384 cloacal swabs and 142 feather tissues) collected from the field were pooled into 43 eppendoff tubes according to the farm/household where samples were obtained from. Thirty one (31) of these pooled samples were cloacal swabs and 12 were feather specimens. Pooling of the samples was done to ensure optimization of the use of reagents.

3.12 RNA Extraction

Single stranded viral RNA was extracted using the QIAamp® Viral RNA Mini Kit commercially available from QIAGEN (Qiagen, Hilden, Germany). The viral RNA mini spin procedure as recommended by the manufacturer was used and the manufacturer's instructions were followed. 560 µl of lysis buffer containing carrier RNA was added to 140 µl of cloacal or feather tissue homogenate and mixed by pulse vortexing. The mixture was incubated at room temperature for 10 minutes. 560 µl of 96-100% molecular grade ethanol was added and again mixed by pulse vortexing. Six hundred and thirty µl of the mixture was transferred to the QIAmp Mini spin column and spun at 8000 revolutions per minute (rpm) for 1 minute. This step was repeated for the remaining 630 µl of the mixture. The RNA now bound to the membrane in the spin column underwent two washing steps with 500µl of washing buffers designated AW1 and AW2 to remove all contaminants. Bound RNA was then eluted in 60µl of a special RNase – free buffer into DNAse/RNAse free tubes. Extracted RNA was stored at -30^{O}C until use. The volume of RNA used as template for RRT-PCR was 8µl.

3.13 Real-Time Reverse Transcription-PCR PLATE SETUP

The RT-PCR plate setup for both protocols; Spackman *et al*, 2002 and CDC protocol (WHO, 2009) was the same and as follows: the Negative and Positive Controls were distantly placed (A1 and H12 wells of the plates respectively) to avoid contamination. The field samples were placed systematically from the A4 well to C9 well of the PCR plate.

3.14 Real-Time Reverse Transcription-PCR (Spackman) testing of Samples

The presence of Influenza A Matrix gene in the 526 samples was determined at the Virology Department (P3 laboratory) of the NMIMR using rRT-PCR protocols developed by Spackman *et al.*, 2002 and CDC Atlanta USA, 2007.

Spackman *et al* Protocol: The Qiagen one-step RT-PCR kit was used with a 20µl reaction mixture under the following conditions: 0.8 µl of kit supplied enzyme mixture (including RT and hot-start *Taq* polymerase), 10 pmol of each primer, 0.3 µM probe, 400 µM (each) dNTPs, 3.75mM $MgCl_2$ and 6.5U of RNase inhibitor (Promega, Madison, Wisconsin). Reverse transcription was achieved at $50^{O}C$ for 30minutes .Taq polymerase activation was at $90^{O}C$ for 15 minutes. A two-step PCR cycling protocol was then used for the matrix gene primer and probe set as follows 45 cycles of $94^{O}C$ for 0seconds for denaturation and $60^{O}C$ for 20seconds annealing. RRT-PCR was performed with the Applied Biosystems Incorporated (ABI) 7300 system thermocycler and software.

3.15 Confirmation of results using Real-Time Reverse Transcription-PCR, CDC Protocol (WHO, 2009)

The negative results obtained using the Spackman protocol were confirmed as described by the Centres for Disease Control and Prevention, Atlanta USA in their protocol CDC Real-time RTPCR (rRT-PCR) Protocol for Detection and Characterization of Influenza (version 2009) using reagents from the Invitrogen One-Step Superscript III RT-PCR kit. Primers designed and supplied by the CDC were used at a concentration of 20 pmol each in a 25 µl reaction mix with 0.5µl kit supplied enzyme, 0.25µM probe (designed by the CDC) and 12.5µl of kit supplied 2X reaction mix, a buffer containing 0.4 mM of each dNTP, 2.4 mM MgSO4. Cycling conditions

Table 3: PCR Master Mix Formula (Spackman *et al.*, 2002)

MASTER MIX	VOLUME (µl)	VOLUME/TUBE	TOTAL
Water	3.1	3.1	155
5 x Buffer of Qiagen kit	*4*	*4*	*200*
MgCl (Promega)	1	1	50
dNTPs	0.8	0.8	40
M+64(FAM-TAMRA) probe	0.3	0.3	15
M+25 Primer	0.5	0.5	25
M+124 Primer	0.5	0.5	25
ROX dye working dilution (1:100)	1	1	50
Qiagen one-step Enzyme Mix	0.8	0.8	40
		12	**600**
Template Volume	8	Number of tubes: **50**	
Reaction Volume	**20**		

Table 4: PCR Primer and Hydrolysis Probe Sequence for AI Virus Detection (Spackman)

Specificity	Primer/probe	Sequence "(5'-3')
Influenza A Matrix	M + 25	AGA TGA GTC TTC TAA CCG AGG TCG
	M - 124	TGC AAA AAC ATC TTC AAG TCT CTG
	M + 64	*FAM-TCA GGC CCC CTC AAA GCC GA-TAMRA*

FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetralrhodamine. Source : Spackman *et al* 2002

Table 5: Amplification Cycling (Spackman)

RT Reaction	Starting	Denaturation	Annealing
50°C	94°C	94°C	60°C
30 min	15 min	0 sec	29 sec
		45 cycles	

Table 6: PCR Master Mix Formula (CDC Protocol)

MASTER MIX	VOLUME (µl)	VOLUME/TUBE	TOTAL
Nuclease Free Water	5.0	5.0	230
2 x Reaction Mix	12.5	12.5	575
Probe (FAM-TAMRA)	0.5	0.5	23
Forward Primer	0.5	0.5	23
Reverse Primer	0.5	0.5	23
25 x RT-PCR Enzyme	1.0	1.0	46
Total		**20**	**920**
Template Volume	**5.0**	5.0	Number of tubes: 46
Reaction Volume	**25**		

Table 7: Amplification cycling (CDC Protocol)

RT Reaction	Starting Denaturation	Denaturing	Annealing
50°C	95°C	95°C	55°C
30 min	2 min	15 sec	30 sec
		45 cycles	

3.16 DATA ANALYSIS/PROCESSING

Both quantitative and qualitative data obtained from the questionnaires and data from the RRT-PCR testing was double entered into Epidata Software (2007 version) and coded accordingly. This was then exported to SPSS Software version 17.0 for analysis. Checks were performed by running simple frequencies to clean and reconcile data. Because the test was negative for all the samples (526 cloacal and feather tissues), the dependent variables (RRT-PCR test results) could not be used against the independent variables which consisted of the farmers' attitude (adherence or non-adherence) towards biosecurity and farm management practices. Eventually, data was analyzed into percentages and tables based on adherence or non-adherence of each farm premises to the 19 independent variables or biosecurity practices (Tables 11a-11c) investigated in this study. Test of association (chi-square) was also performed on certain variables (age and sex) to determine whether they deferred significantly.

CHAPTER FOUR

RESULTS

4.1 DESCRIPTIVE CHARACTERISTICS OF BIRDS SAMPLED

Apart from been raised for meat and eggs, some ducks in the area were also kept as pets or for ornamental value. It was observed that the ducks in the area were mostly hybrids from the *Anas platyrhynchos domesticus* family particularly the Mallards. Eighty four percent (323/384) of the ducks sampled were hybrids; the remaining 16% (61/384) were made up of thorough Mallard breeds (Aylesbury, pekin and pennine).

Out of the 526 samples collected, 58.6% (308/526) were from backyard holdings, 34% (179/526) from a commercial farm and 7.4% (39/526) from a live birds market (LBM).

Seventy six percent (292/384) of the ducks were females (ducks) and 24% (92/384) were males (drakes). Adult ducks (> 1 year old) represented 79.9% (307/384) of the sampled population while growers or ducklings (< 1 year old) formed only 20.1% (77/384) of the same population. The number of ducks of age greater than 1 year compared to those less than 1 year was found to be statistically significant at $p<0.0001$. The same was for male and female ducks sampled (Tables 9 and 10).

The male to female ratio in breeding pens was found to be 5-8 females (ducks) to a male (drake).

Table 8: Details of samples collected from ducks in the Sunyani Municipality (July 2009 - October 2010).

Farm Number	Farm Size	Quantity of Samples Collected		Location of Farm
		Cloacal swabs samples	Feather	
1*	1,113	119	60	Dumasua
2	25	24	7	New Dormaa
3	12	12	7	New Dormaa
4	23	21	7	New Dormaa
5	241	57	20	Abesim
6**	38	30	10	New Dormaa
7*	44	32	7	Sunyani LBM
8	78	46	17	Odomase
9	75	43	7	Adantia
Total	1,649	384	142	----------

* Farm numbers 1 and 7 are; a Commercial Farm and a Live Birds Market (LBM) respectively. The remaining seven are backyard holdings. ** The only duck farm sampled that experienced outbreak in 2007.

Table 9: Sex Distribution of Ducks by Farm/Household in Sunyani Municipality (July 2009 – August 2010).

Farm Number	Male		Female		Total	
	Number	%	Number	%	Number	%
1	25	20	100	80	125	100
2	5	27.8	13	72.2	18	100
3	3	25	9	75	12	100
4	6	28.6	15	71.4	21	100
5	16	28.1	41	71.9	57	100
6	8	26.7	22	73.3	30	100
7	11	34.4	21	65.6	32	100
8	10	21.7	36	78.3	46	100
9	8	18.6	35	81.4	43	100
Total	92	24	292	76	384	100

The above Table shows the number of males and female ducks in each of the nine sites studied. The calculated *Pearson* Chi-square was 5.195 with a $p < 0.0001$. The difference was found to be statistically significant at 99.9% CL in all the nine sites.

Table 10: Age Grouping of Ducks Sampled in the Sunyani Municipality, (July 2009 – August 2010).

Farm Number	Ducks greater than one year old		Ducks less than 1 year old		Total	
	Number	%	Number	%	Number	%
1	94	75.2	31	24.8	125	100.0
2	5	27.8	13	72.2	18	100.0
3	12	100.0	0	0.0	12	100.0
4	15	71.4	6	28.6	21	100.0
5	54	94.7	3	5.3	57	100.0
6	30	100.0	0	0.0	30	100.0
7	26	81.3	6	18.8	32	100.0
8	40	87.0	6	13.0	46	100.0
9	31	72.1	12	27.9	43	100.0
Total	307	79.9	77	20.1	384	100.0

The above Table shows the number of adult ducks (age > 1 year) and that of ducklings (age < 1 year) in each of the nine sites studied. The calculated *Pearson* Chi-square was 54.501 with a $p < 0.0001$. This was found to be statistically significant at 99.9% CL in all nine cases.

4.2 SOURCES OF PARENT STOCK

Sources of ducks in all the sites were identified. The commercial farm acquired parent stock from a farm in Dormaa Ahenkro in 2005 (Anonymous). Our findings also indicated that 71.4% (5/7) of the backyard holdings acquired their birds from live birds markets near and far. We also found that various species of birds in the live birds market investigated came from sources within and outside the municipality. Birds were purchased by customers for different purposes such as consumption (household, and restaurant), offering in ceremonies/gifts and religious festivals, and for replacement stock for farmers.

The study further showed that transportation and management of birds by market vendors involved poor biosecurity practices. Collectors (intermediary men) and vendors did not separate birds according to species and sources of birds. Also, birds were mixed in cages during transportation and at the market place.

4.3 MORBIDITIES AND MORTALITIES IN DUCKS AT STUDY SITES.

The study revealed that three months preceding this study, 55.6% (5/9) of the sites investigated experienced increased illnesses in birds; however mortalities were recorded in 44.4% (4/9) of the sampled sites (Table 10). The overall mortality rate was calculated as 7.3% with a p-value of 0.7 at 95% confidence level. Signs and symptoms documented to have been exhibited by these sick birds were not compatible with those of classical HPAI H5N1 infection described by Capua and Mutinelli, 2001.

Nonetheless, it is important to note that though HPAI H5N1 viruses are 100% lethal for chickens and other gallinaceous poultry, some ducks species do not show signs and symptoms of the disease after been exposed (Swayne D and Beck J 2005; EC 1992 amended 2004).

Table 11: Mortality Rates of ducks in the various sites in Sunyani Municipality (April 2009– June 2009).

Farm Number	Location	Flock Grouping	Mortality rate (%)
1	Dumasua	Commercial	1.4
2	Asuokwa	Backyard	-
3	Asuokwa	Backyard	-
4	Asuokwa	Backyard	41.6
5	Abesim	Backyard	-
6	Asuokwa	Backyard	-
7	Sunyani Market	Live birds market	11.4
8	Odomase	Backyard	-
9	Adantia	Backyard	11.5

Mortality rates shown on the above table were calculated using deaths that occurred in some of the samples collection sites prior to the study. Cause(s) of death were unknown.

4.4 BIOSECURITY AND POULTRY MANAGEMENT AT STUDY SITES

Semi-intensive and free-range were generally observed to be the management systems practiced in all the sites where samples were collected for the study. In the semi-intensive also known as semi-confinement, we observed that the birds were fenced in, or restricted to a yard and were mostly fed with damaged grains, food left-overs and less commonly with self-prepared poultry feed. The only commercial duck farm investigated, fed their birds with either commercially prepared feed or self-prepared feed (including whole and damaged grains). On the other hand, in the free-range or open housing system, ducks were allowed to roam freely within the farmer's property, and on those of their neighbours. They were left to scavenge for feed with little grain supplements offered them. Sixty six point seven percent (6/9) of the sites sampled were semi-intensive (with perimeter fencing) while the remaining 33.3% (3/9) were opened or free-range.

Biosecurity which is a set of management practices which reduce the potential for the introduction and spread of disease-causing organisms onto and between poultry sites was not duly observed in totality by all the farm premises in this study. Our investigation revealed that 89% (8/9) of the sites investigated did not adhere to strict biosecurity and farm management practices. The only commercial farm complied with fifteen out of the 19 different practices observed in the study (Tables 11a-11c). We also found out that though 33.3% of the sites practiced free range and therefore had their birds exposed to other birds including wild birds, two other semi-intensive sites had wild birds occasionally invading their premises in search of feed, and this usually bring their birds also into contact with wild birds therefore increasing the number of domestic ducks exposed to wild birds.

Our findings also indicated that none of the sample collection sites practiced all-in-all-out, a basic principle of poultry husbandry which prescribes that only birds of the same species, age and purpose (either broiler, layer etc.) are kept at a time till the end of their life span/production period and the needed processes are followed and a new batch of birds with similar characteristics are introduced to the premises. The study showed that 100% (9/9) of the sites investigated had birds with varied ages (multi-age) and only 33.3% (3/9) of these sites kept only one species (ducks). The remaining 66.7% (6/9) practiced mix-breeding (breeding of different species together).

In this investigation, only 33.3% (3/9) of the sites were found to carry out disinfection regularly in their premises, the other 66.7% (6/9) did it occasionally or not at all.

This study also found out that farm works in 88.9% of the sites were complying with the measures of preventing workers from going from farm to farm. Workers in 11.1% (1/9) of the sites defaulted. Findings in this study also indicated that, only 33.3% (3/9) of the sites had functional drainage system, 22.2% (2/9) practiced personnel disinfection, and workers in 33.3% (3/9) of the sites used protective gears. In 77.8% of the sites investigated, waste generated was thrown away around the premises instead been composted for use as manure. The disposal of dead birds was mostly done by burying in 77.8% (7/9) of the premises while the remaining 22.2% (2/9) practiced burning. Measures such as quarantine of new birds, use of protective gears by visitors and disinfection of vehicles were only practiced in the only commercial farm investigated. The remaining 89% (8/9) of the sites did not patronize these measures. The study also revealed that water delivery and feeding of birds were done manually (not automated) in all nine sites. At the live birds market, vendors did adhere to any of

the biosecurity or management practices except sweeping the floor around their cages and occasional mechanical scrubbing of the cages.

Table 12a: Frequency of Bio-security and Husbandry Practices among Farmers at Study Sites, Sunyani Municipality (July 2009 – October 2010).

Activity		Number of Premises	%
Type of Housing	Free range	3	33.3
	Closed	6	66.7
Perimeter Fencing	Yes	6	66.7
	No	3	33.3
Drainage System	Yes	3	33.3
	No	6	66.7
Daily disinfection of premises	Yes	1	11.1
	No	8	88.9
Use of protective clothings by workers	Yes	3	33.3
	No	6	66.7
Use of protective clothings by visitors	Yes	0	0.0
	No	9	100.0
Isolation of sick ducks	Yes	4	44.4
	No	5	55.6
Quarantine of new entry birds	Yes	0	0.0
	No	9	100.0
Disinfection of vehicles	Yes	0	0.0
	No	9	100.0

Table 12b: Frequency of Bio-security and Husbandry Practices among farmers at Study Sites, Sunyani Municipality (July 2009 – October 2010).

Activity		Number of Premises	%
Personal disinfection	Yes	2	22.2
	No	7	77.8
Contact with wild birds	Yes	5	55.6
	No	4	44.4
Deworming/antibiotic use	Yes	6	66.7
	No	3	33.4
Cleaning of feeders/waterers	Daily	3	33.3
	Not daily	6	66.7
Disposal of dead carcasses	Buried	7	77.8
	Burnt	2	22.2
Waste management	Thrown away	7	77.8
	Use for manure	2	22.2
Type of breeding	Ducks only	3	33.3
	Ducks and others	6	66.7
Management practices	All-in-all-out	0	0.0
	Multi-age	9	100.0
Availability of footbaths	Yes	0	0.0
	No	9	100.0

Table 12c: Frequency of Bio-security and Husbandry Practices among Farmers at Study Sites, Sunyani Municipality (July 2009 – October 2010).

Activity		Number of premises	%
Feed	Self-prepared	6	66.7
	Scavenging for feed	3	33.3
Water Delivery	Automated	0	100.0
	Manual	9	0.0
Feeders	Automated	0	100.0
	Manual	9	100.0

4.5 MOLECULAR INVESTIGATION FOR AVIAN INFLUENZA

Two different RT-PCR protocols (Spackman *et al.*, 2002 and the CDC Real Time PCR protocol) were applied on 526 ducks samples (384 cloacal swabs and 142 feather tissues) to determine the presence of AI virus in ducks. All the samples were negative for AI virus. This included ducks sampled from the only duck farm in the Sunyani Municipality which was affected by the 2007 HPAI H5N1 outbreaks. This farm was closed down as a result of the outbreak in May 2007 and became operational three years ago with current population of 38 ducks (Table 8).

Table 13: Results of RRT-PCR Tests for AI conducted at the NMIMR Virology Department using both Spackman *et al.*, 2002 and CDC Protocols (WHO, 2009), between October 2010 and April 2011 respectively.

Sample	Target Gene	Cycle Threshold (C_t)
Positive Control	Flu-A Matrix Gene (Qiagen)	27.50/20.50
Negative Control	,,	Undetected
Commercial Ducks Samples	,,	Undetected
Backyard Ducks Samples	,,	Undetected
Live Birds Market Samples	,,	Undetected

"Undetected"= Negative results. The 27.50 cycle threshold value recorded for the "Positive Control" is a Positive result. The cut-off point for this test was ≤ 35 c_t value.

Fig 8: Real-Time PCR (Spackman *et al* 2002) Amplification Plot for AI Virus Detection in the 43 Pooled samples, April 12, 2011.

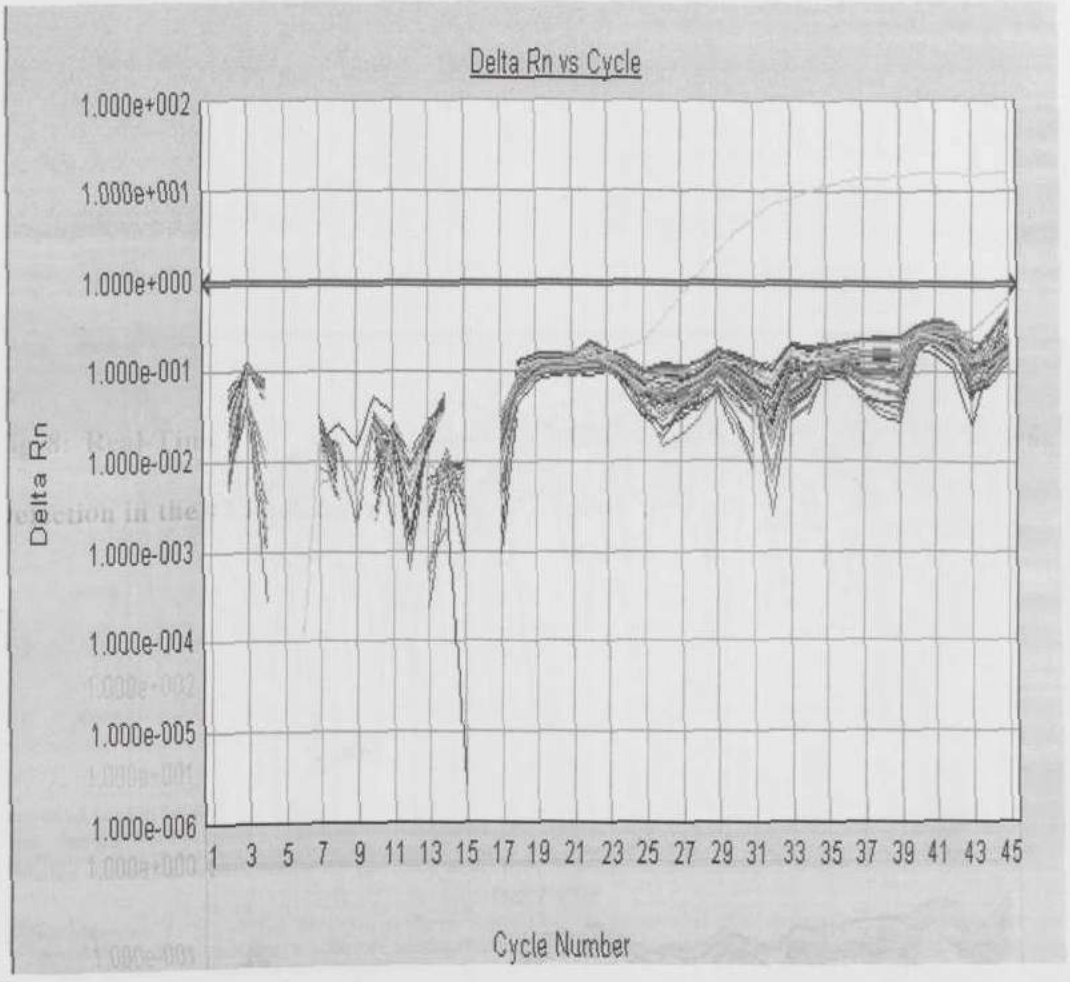


Figure 5 shows RRT-PCR amplification plot for Flu A Matrix Gene analysis for the 43 pooled samples analyzed using the Spackman *et al.* (2002) Protocol. The Positive Control (green line above baseline) increased exponentially with a cycle threshold (C_T) value of **27.50**. The Negative Control and the 43 pooled samples were undetected by the system's software. The cut off point for the test was a threshold ≤ 35 C_T value. The RRT-PCR lasted for less than three hours with 45 cycles.

Fig 9: Real-Time PCR (CDC Protocol) Amplification Plot for AI Virus Detection in the 43 Pooled Samples, October 17, 2010.

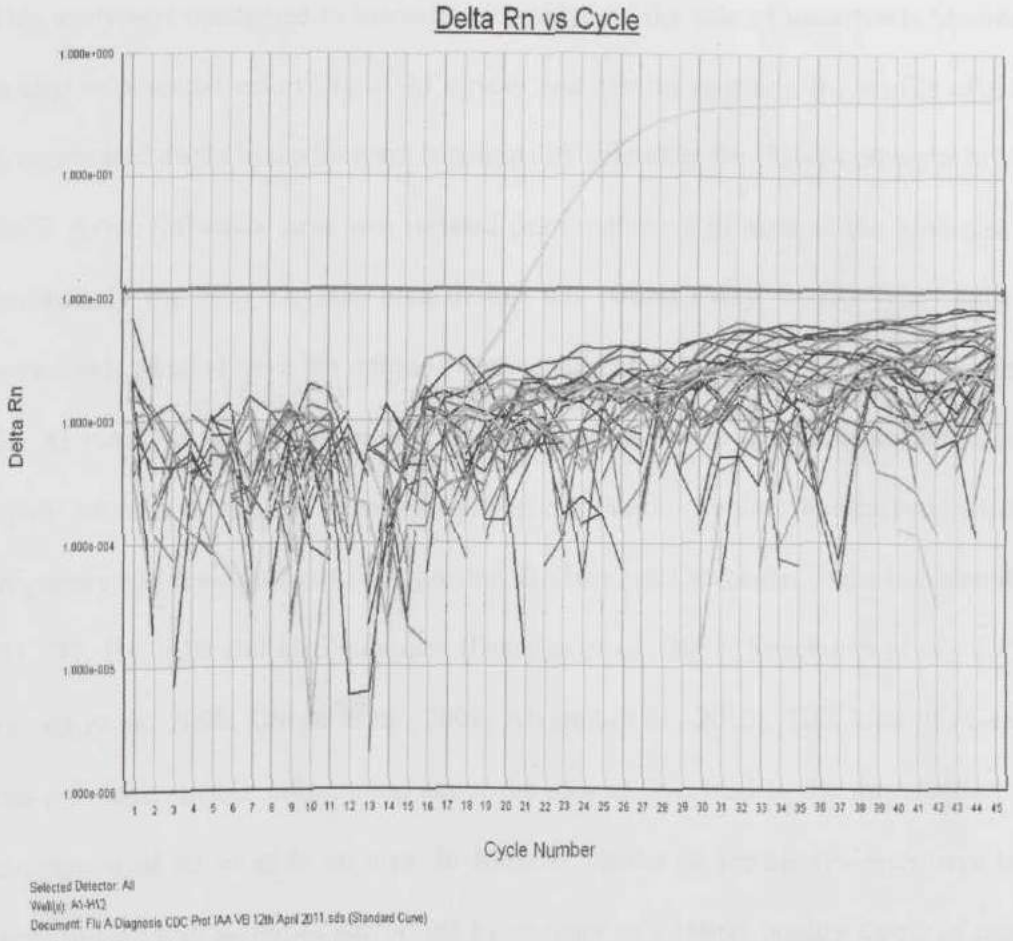


Figure 6 displays the RRT-PCR amplification plot for Flu A Matrix Gene analysis for the 43 pooled samples analyzed using the CDC Protocol. The Positive Control (green thin line above baseline) increased exponentially with a C_T value of **20.50**. The Negative Control and the 43 pooled samples were undetected by the system's software. The cut off point was a threshold ≤ 35 C_T value. The test lasted for less than three hours with 45 cycles.

CHAPTER FIVE

DISCUSSION

This study was conducted to increase knowledge on the role of waterfowls (domestic ducks) as potential reservoirs of AI viruses and also to ascertain the profile of AI in domesticated ducks in the Sunyani Municipality area after the H5N1 outbreaks in May 2007. Avian influenza virus was isolated from outbreaks in parts of the Municipality particularly the New Dormaa area during this period (May, 2007). Also, because waterfowls (ducks) have the unique characteristic of being asymptomatic reservoir of the AI virus, we embarked on this study in the area where there was an outbreak to verify whether or not the virus was still in circulation. Similar studies conducted in migratory and resident birds in Argentine, Bolivia and Caribbean countries, identified H1, H3, H4, H10 and H13 subtypes (Douglas *et al.*, 2007; Spackman *et al.*, 2007a; Pereda *et al.*, 2008; Ghersi *et al.*, 2009; Alvarez *et al.*, 2010). This kind of research can provide valuable information about the species involved in the introduction and circulation of AI virus in an area. In Mexico, results of similar research have been incorporated into activities performed by sanitary officials at poultry farms as part of the campaign for early detection and prevention of HPAI outbreaks (Villarreal-Chavez and Rivera-Cruz, 2003). This could be replicated in Ghana.

The results obtained from the present study showed no evidence of the presence of AI virus in the five hundred and twenty six (526) AI samples collected from domestic ducks in nine farms in the Sunyani Municipality. These samples were tested using two different RT-PCR protocols; Spackman *et al.*, (2002) and CDC protocol (WHO, 2009). Polymerase Chain Reaction which was the test used has been used as the new

gold standard for detecting a wide variety of templates across a range of scientific specialties including virology (Freymuth *et al.*, 1995). Furthermore, standard RT-PCR and real-time RT-PCR methods have been applied to the detection of avian influenza virus in several instances (Lee S *et al.*, 2001; Munch *et al.*, 2001 and Spackman *et al.*, 2002). Hence, the fact that two different "tried and tested" RRT-PCR protocols were applied in this study and both tested negative for the virus in all the samples probably confirmed the validity of the results obtained and therefore suggest that there is currently no circulation of the virus in the area. Also, the fact that we used feather tissues which are known for shedding large quantities of viral particles which can persist for longer periods after detaching from the body (Yamamoto *et al.*, 2008) is another prove that the results is valid and therefore reliable. A similar study carried out in commercial, backyard and live birds market in the Tema Metropolis where there have been outbreaks (May, 2007) from May 2009 to September 2010 also yielded negative results (Danso E.F *et al.*, 2010). The present negative results obtained also included farm number 6 (See Table 8) which was the only duck farm where there was an outbreak in 2007 and birds (ducks) were destroyed and containment measures put in place.

Therefore, the results obtained is an indication that the measures that were put in place in affected farms by the Veterinary Services Directorate in the 2007 AI outbreaks (VSD Annual Report, 2009) which included depopulation of affected farms, decontamination of premises, enforcement of rest period for affected farms, education of stakeholders on biosecurity among others yielded the desired results. However, evidence gathered by the present study pointed to deterioration of farm management practices particularly biosecurity, and if left unattended to and reintroduction of AI

virus occurs, the effect may be more disastrous to the poultry industry than the 2007 outbreaks, with possible human incursions. We observed in our study that biosecurity which is the cornerstone in poultry disease prevention and containment has in many instances been compromised. For instance, we observed that 89% (8/9) of the sites where samples were collected from did not adhere to best biosecurity practices. Because 33.3% (3/9) of the study sites had ducks in free-range, they roamed around freely interacting with different species of animals including wild birds which could be harbouring the virus and therefore posed threat to these ducks getting infected with the virus and spreading it. Also, mingling of different species of livestock could result in serious public health issues if the virus is present and reassortment takes place particularly in the pig.

Irrespective of the size of a poultry farm, bio-security measures include fencing or walling of farms, provision of footbaths and vehicle dips with constant replenishing of disinfectant solution. Other measures include use of protective clothings by farm workers and visitors, personnel disinfection, disposal of dead birds, disinfection of premises, quarantine of new birds and others. In this study we found out that biosecurity practices which had to do with the use of disinfectants (disinfection of premises 11.1%, personnel disinfection 22.2%, use of footbath 0% and disinfection of vehicles 0%) were considered expensive hence were either not practiced or were less practiced compared to those that were not disinfectant dependent. This was also observed by Danso and others (2010) in their findings. It is true that these so called expensive activities required recurrent expenditure especially the frequent use of chemicals which are expensive.

Activity such as delivery of water and feed to birds could act as a source of contamination which could enhance the spread of poultry diseases when being administered manually without proper hygienic practices. According to Adak G.K *et al.* (1995) and Rodriguez *et al,* (2001), contaminated food and water are believed to be major sources of infection in warm-blooded animals including poultry. That is why in modern poultry husbandry the use of automated system of feed and water delivery to birds instead of the old aged manual delivery is preferred. In this study however, our findings indicated that none of the sites practiced automated feeding or watering. This therefore put all these sites at a potential risk of various pathogens including the AI virus. Also, quarantine of birds which is one surest way of making sure new birds carrying active or passive infections do not come into contact with the old flock, was not adhered to in any of the sites studied. Infact, non-adherence to this practice is extremely dangerous to poultry production because of its potential to trigger and maintain the spread of new infectious agents including the HPAI H5N1 in a healthy flock. Effective cleaning and disinfection reduces pathogen numbers and the weight of disease challenge, and enhances biosecurity programme in a poultry setting. However, our study revealed an awfully low patronage (11.1%) for cleaning and disinfection of premises. Also, visitors to these premises did not put on protective gears to avoid been infected by sick birds or to ensure they did not introduce pathogens to farm premises. Again, the study showed that none of the sites investigated practiced this very important biosecurity measure (donning of PPEs by visitors). Another equally important biosecurity measure was the disinfection of vehicles to and from the farm premises which was never observed in any of the sites. Though there was no available data on the biosecurity situation in the area prior to this study, it was clear that many farms (89%) did not still adhere to strict biosecurity and farm management practices.

At the live birds market, vendors did not adhere to any of the requirements of bio-security except for daily sweeping. Birds were slaughtered right there and dressed in unsanitary conditions. Also, the dangerous practice of mixing different species of birds in the same cage and also mixing sick and healthy birds was evident. Some of these apparently sick birds were placed outside the cage in the mist of other market vendors and buyers. There was therefore an apparent public health threat because birds purchased were sent to near and far for different purposes such as for consumption (at homes, restaurants etc), rearing and others, and if these birds are infected they could perpetuate the spread of the disease in birds and other animals including humans. There is therefore the need to establish effective biosecurity barriers between the LBM, Commercial/Backyard poultry and humans in the municipality.

In this study, the difference in sex amongst the ducks investigated was found to be statistically significant with a p-value of 0.0001 at 95% confidence level, and the accepted male/female ratio of ducks in breeding according to Koney (1998) is 1 male to 5-8 females. However, the male/female ratio in this study was found to be 1:3 which suggested that there could be in-fighting among males in mating females since there was deviation from the norm (1 male to 5-8 females). This difference could then encourage males (those in free-range) to migrate to other territories in search of females hence favoring the spread of diseases amongst birds.

Non-specific deaths (agent-s unidentified) of ducks were recorded in four of the study sites three months before we embarked on samples collection. An overall mortality rate of 7.3% was observed amongst ducks in the study sites with a p-value of 0.7 at 95% confidence level which was found not to be statistically significant. Hence,

backed with the fact that signs and symptoms documented to have exhibited by these sick birds were not compatible with those of classical HPAI H5N1 infection (Capua and Mutinelli 2001), H5N1 virus infection was not suspected. However, because high mortality rates were recorded in two backyard holdings and the live birds market (Table 10) which were characterised by non-adherence to biosecurity practices, we suspected possible deficiencies in biosecurity to be the cause of the deaths.

5.1 STUDY LIMITATION/CONSTRAINT

An equal number of feather tissue samples and cloacal swabs could not be collected due to lack of Viral Transport Medium (VTM). Also, viral isolation method which is a gold standard test for AI diagnosis was not applied.

CHAPTER SIX

CONCLUSIONS AND RECOMMENDATIONS

6.1 CONCLUSIONS

There was no evidence of AI virus in domestic ducks in the Sunyani Municipality. Hence domestic ducks in the Municipality are not acting as reservoir of AI viruses. However, adherence to strict farm management and biosecurity practices was not observed by 89% of the sites investigated. Five (66.7%) out of the nine sites including the commercial farm had their premises fenced or walled. The remaining backyard holdings (33.3%) were free-range. Multi-aged and different species of birds (100% and 66.7% respectively) were kept together including the live birds market (LBM). The practice of all-in-all-out never practiced in any of the sites. The practice of quarantining new birds, use of protective cloths by visitors to farm premises, vaccination of birds against diseases and provision of footbaths for dipping vehicular and human traffic was not practiced in any of the sites investigated. Generally, ducks farmers in the Municipality had little knowledge about biosecurity practices.

Sixty four percent of the ducks involved in this study were hybrid strains bred for both meat and egg production. Also, majority 79.9% of them were greater than one year (> 1 year) old compared to the 20.1% which were less than (< 1 year) old. All the sites had more female ducks than males (drakes). Fifty five point six percent of the ducks investigated had contact with wild birds.

Most backyard holdings 71.4% acquired their ducks from live birds markets (LBMs) . Hundred percent of the sites investigated used manual feeders/waterers instead of

automated. Biosecurity practices which depended on the use of disinfectants were less adhered to compared to those that did not depend on disinfectants

Also, deep burying of dead birds and burning were the most common methods of disposal of dead birds practiced though burying was most preferred (77.8% of the sites). Daily sweeping of the premises was the major sanitation practice in the LBM involved in this study.

The time period for the study was limited hence, continuous surveillance will be necessary for early detection and response. Also, we failed to conduct viral isolation, the gold standard test for AI diagnosis.

Finally, we have no doubts that the investment made in terms of time, finance and material resources in undertaking this study is worth it. It is our believe therefore, that the proposed recommendations indicated below if carefully considered and implemented to the letter will go a long way to minimize the reintroduction of avian influenza and facilitate containment should the unfortunate situation of reoccurrence becomes a reality.

6.2 RECONMMENDATIONS

i. The Veterinary Services Directorate (VSD) should continue with both active and passive surveillance of AI disease in ducks and other poultry especially in high risk areas such as the 2007 outbreak areas, wetland sites, live birds markets and border communities all over the country.

ii. VSD should intensify bio-security, poultry management and sanitation awareness creation among farmers and the public.

iii. Now that some studies have been carried out in the Tema Metropolis and Sunyani Municipality, a similar survey in Aflao in the Volta region which also experienced outbreaks in 2007 should be considered by SPH, NMIMR and VSD.

iv. VSD should equip the local veterinary clinic in Sunyani with AI rapid diagnostic kits and train veterinary staff in tentative diagnosis of the disease at pen sides.

v. Ministry of Food and Agriculture (MOFA) and VSD should encourage Field Epidemiology and Laboratory Training Programme residents to replicate this study in these high risk areas.

vi. Viral isolation as a diagnostic tool should be considered in future studies.

vii. MOFA should equally subsidize the cost of disinfectants for poultry farmers as its being done with fertilizer.

viii. The Sunyani Municipal Assembly (SMA) should enact by-laws that would prohibit loitering of poultry and other animals around the city to avoid mingling of domestic birds and other animals particularly wild birds which could serve as source of AI virus.

ix. SMA should also construct modern live birds markets outside the main markets to avoid spread of diseases to humans from infected birds.

x. Farmers should be trained on AI disease recognition and be encouraged to report promptly to the Veterinary any unusual signs in their birds including unexpected deaths among birds.

xi. Farmers should be trained on biosecurity practices as outlined in the Biosecurity Manual prepared by Veterinary Services Directorate of Ministry of Food and Agriculture for training of field staff, poultry farmers and other stakeholders.

xii. VSD should monitor poultry farms to ensure the biosecurity practices learnt are adequately applied.

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APPENDICES

8.1 PARTICIPANTS CONSENT FORM

Study title: Domestic Ducks as Potential Reservoirs of Avian Influenza Virus in H5N1 Outbreak Area, Sunyani Municipality.

Principal investigator: Dr. Vitus Burimuah

Organization: Veterinary Services Division of Ministry of Food and Agriculture/ FELTP-Ghana.

General Information about Research

We do recall that in 2007 there were two confirmed outbreaks of avian influenza (AI) in poultry in the Sunyani municipality. Because ducks are known to be asymptomatic carriers of AI, this study is intended to determine whether or not the AI virus that entered the area is circulating discreetly in ducks population in the area. Information obtained from the study will guide in the adoption of relevant surveillance strategies needed to prevent recurrence of AI outbreaks in Ghana.

Participants are expected to collaborate with the research team during the sample collection period which has one month duration. We expect prospective participants to cooperate fully with the team by making available their ducks on time to ensure speedy work. Samples to be collected from the ducks will include cloacal swabs and feather calamuses (1 per duck). Participants will also be asked questions regarding poultry management, heath status of birds, and movement of farm equipments, vehicles and the use of personnel protective equipment (PPE) by farm workers/ visitors.

Possible Risks and discomfort There are hardly any risks associated with your participation in this study.

Possible Benefit: The benefit of taking part in this study is that if you have any questions concerning Avian Influenza, we will try to answer them. You would also be contributing to knowledge that is useful for the successful control of AI infection in Ghana.

Compensation: You will not be given any incentives to take part in the study.

Confidentiality: Any information about you that will be collected during the study will be confidential and will be stored in a file which will have only a number assigned to it and not your name.

Voluntary Participation and Right to Leave the Research: Your participation in this study is purely voluntary and you are free to withdraw at any point in the study. You would not suffer any penalty for refusing to participate or for withdrawing from the study at any point

Contacts for Additional Information

If you have any questions about your rights as a study participant or about ethical matters please contact the Chairman, Institutional Review Board of the Noguchi Memorial Institute of Medical Research, at phone number 021 501178/9.

For general questions about the study you can call the principal investigator:

Vitus Burimuah, Department of Epidemiology and Disease Control, School of Public Health, University of Ghana, Legon.

Your rights as a Participant

This research has been reviewed and approved by the IRB of Noguchi Memorial Institute for Medical Research. If you have any questions about your rights as research participant you may also contact Rev. Dr. Ayete-Nyampong, Chairperson, NMIMR-IRB, mobile 0208152360.

CONSENT FOR STUDY PARTICIPATION

The above document describing the benefits, risks and procedures for research of ducks as potential reservoirs of avian influenza viruses in post HPAI H5N1 outbreak area, Sunyani municipality has been read and explained to me. I have been given an opportunity to have any questions about the research answered to my satisfaction. I agree to participate as a volunteer.

_____ _____

Date Signature or mark of volunteer

If volunteers cannot read the form themselves, a witness must sign here:

I was present while the benefits, risks and procedures were read to the volunteer. All questions were answered and the volunteer has agreed to take part in the research.

_____ _____

Date..... Signature of witness

I certify that the nature and purpose, the potential benefits, and possible risks associated with participating in this research have been explained to the above individual.

_____ _____

Date Signature of Person Who Obtained Consent

8.2 QUESTIONNAIRE

GENERAL INFORMATION

Date: Investigator: Samples obtained:

....
.....

Farm Name:.....Location:.....Farm Owner(s):

Birds kept at other location: GIS co-ordinates:.....

.....

Farm Contact (Phone#):.....

LIVESTOCK DATA

Farms activities: ☐ Ducks ☐ Chickens ☐ Others

Check all that apply

Commercial type/species: Males (>1yr) Females (>1yr).....Age range Total

....
.....

Non-Commercial types/species: Males (>1yr) Females (>1yr) Age range.....Total

....
.....

Game fowls:.....

Psittacins:.....

Waterfowls:.....

Contact with wild bird species: Yes ☐ No ☐ describe.....

List other animals species located on the premises:

Bird Increased illness in birds previous 3 months? Yes☐ No ☐

Number sick: Last week.....Last month.....Last 3 months.....

Number dead: Last week.....Last month..... Last 3 months.....

Vaccination Practices:

Vaccine Use and number of birds vaccinated
Frequency

....
.....

Medication used Use and number of birds treated Dates

....
.....

Birds introduced to the premises in the past 90 days?

Type of birds Location where birds came from:

.....
.....

Birds leaving the premises in the past 90 days?

Type of birds: Location where birds went to:

.....
.....

Of birds that left did any return to the premises? Yes ☐ No ☐

If yes,

Type of birds: Location where birds went to:

....

MANAGEMENT AND HUSBANDRY

Type of house(s): Open ☐ Closed ☐ Other ☐

Check all that apply

House materials: Wood ☐ Cement ☐ Metal ☐

Disinfection of houses: Yes ☐ No ☐

Describe.....

Type of husbandry:

Check all that apply All in/All out ☐ Multi-age ☐ Modification/Other ☐

Distance between poultry houses: ☐ to residential area ☐

☐ to nearest poultry farm:..... ☐ to nearest live bird market

Feed: Feed delivered to farm? Yes ☐ ☐ No

Feed stored on premises Yes ☐ No ☐

Workers: Number of workers.....Number of workers per house:.....

Work on other farm? Yes ☐ No ☐

Other questions:

☐ Perimeter fence isolated **Feeders** ☐ Sick birds

☐ Drainage system Automatic ☐ Quarantine of new birds ☐

☐ Personnel disinfection Manual ☐ Disinfection of vehicles ☐

Water delivery

☐ Use of PPE for workers ☐ Automatic Manual ☐

☐ Use of PPE for visitors ☐ Individual entry and traffic Group ☐ Controlled

☐ Poultry house disinfection ☐ Cleaning of feeders/waterers

Frequency:.....

Disposal of dead birds: ☐ Buried ☐ Burned

☐ Use of poultry house rest period ☐ Waste management

Duration:..... ☐ Thrown away ☐ other

If yes, how handled?

SAMPLE COLLECTION

☐ Samples collected previously

Sample type (circle all that apply):

Blood/cloacal

swab/feces/feathers/organ

Results if known.....

☐ Samples collected this visit

List of samples by ID number and type

.....

.....