SEROPREVALENCE STUDY ON AVIAN INFLUENZA IN RURAL POULTRY OF THAIBINH PROVINCE AND CHARACTERIZATION OF THE ENVIRONMENTAL SURVIVAL OF THE AGENTS INVOLVED

MSc THESIS
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Dedication

The thesis is especially dedicated to my mother who always encourages me to pursue my career. I also dedicate this work to my wife and my son, who have missed me and helped me taking care of my mother during the time I was leaving home for doing my MSc course in Denmark.

Contents

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Copenhagen, 19th January, 2005

Do Quy Phuong

Summary

The study is divided into two sections. Section one comprises 4 chapters and section two contains appendices I and II.

Chapter one of section one gives a literature review on avian influenza viruses, in relation to the work performed, and the three remaining chapters deal with the results, discussion and conclusion of the study. Section two includes two articles referred to as Appendix I and Appendix II. Article I describes a survey on antibody prevalence against avian influenza viruses and the virus circulation in village poultry of Thai Binh province in the north of Vietnam. The testing of the survivability/infectivity of H5N1 avian influenza virus following incubation of virus in brackish, pool and arroyo water at different temperatures is described in Appendix II.

The prevalence of positive H5, H3, and H12 sera against avian influenza was detected in village poultry from five districts represent three different geographical areas of Thai Binh province (Appendix I). Blood and cloacal swab samples were obtained from 587 birds including 379 chickens, 76 ducks and 132 muscovy ducks. Antibody to H5 was observed from chickens, ducks and moscovy ducks with titres ranging from 1log2 to 7log2. Antibody levels in chickens were significantly higher than in ducks and moscovy ducks. There was a significant difference in prevalence of H5 antibody among ducks (77.6%), moscovy ducks (24.1%) and chickens (5.8%) ($p = 0.0001$).

Statistical analysis by Chi-square test revealed that there were no significant difference in the prevalence of positive H5 antisera from chickens, ducks and muscovy ducks kept in single species flocks and in mixed flocks (p-value = 1.6, 0.9, 0.52). There was no significant difference in the prevalence of positive H5 antisera of chickens, ducks and muscovy ducks from coastline, interior and riverside areas. The proportion of chickens with positive H5 antisera kept in an indoor system (21.05%) was significantly higher than in scavenging and backyard systems (2.9%, 1.9%). In contrast to the chickens, the proportion of muscovy ducks with positive H5 antisera in a scavenging system (60%) was significantly higher than in birds kept in-door and in backyard systems (26%, 21%).

H5, H3, and H12 antibodies were presented in the same flock or even the same bird with high prevalence of H5 positive in ducks (77.63%), lower in moscovyduck ducks (24.06%) and lowest in chickens (5.82%). In contrast, the seroprevalence of H3 and H12 antisera was higher in chickens (36.2%, 30.6%) and lower in ducks (10.5%, 14.4%) and moscovy ducks (17.2%, 5.2%).

In 587 cloacal swab samples, only one H12 AIV was isolated from a muscovyducks by inoculation to embryonated chicken embryos (ECE) 9 – 11day old.

These finding revealed that H5, H3 and H12 avian influenza viruses have circulated in village chickens. High seroprevalence of H5 antibody was detected in ducks, consequently, ducks could play a role as reservoir for village poultry. Further investigations on the seroprevalance of all AIV subtypes should be performed in future studies.

The survivability/infectivity of H5N1 avian influenza virus was investigated in different types of surface water such as brackish, arroyo and pond waters following incubation at 37ºC, room temperature (20-25ºC) and 4ºC (Appendix II). The virus survived in arroyo water for up to 90 hours at room temperature. In brackish water, the virus lost its infectivity after 6 hours at room temperature. In all three types of water, the virus kept infectivity for 6 hours when incubated at 37ºC and for more than 4 days at 4ºC. When the virus was inoculated into Buffed Phosphate Saline (BPS) 1% milk, it survived more than 4 days following incubation at 37 °C, 20°C - 25°C and 4°C, respectively.

The result of this trial showed that a cool environment is favorable for the survival of H5N1 virus. The virus survived better in water containing organic materials. Furthers studies are needed to determine the maximum survival time in aquatic environments.

INTRODUCTION / BACKGROUND

Chicken production has been documented in Vietnam the last 3200 – 3500 years and it has had a very important role in rural development of Vietnam (Nguyen Dang Vang, 2002). During the last five years the population of poultry in Vietnam has increased rapidly. The total population of poultry is estimated to be 261 million domestic birds including 192 million chickens and 69 millions ducks. The population of geese and ratites is very small and quails and pigeons and other birds are even smaller. The poultry production consists mainly of chickens and ducks raised in backyard household production systems. About 75% of the poultry population is kept in small households (Nguyen Dang Vang, 2002). Village poultry forms an integral part of village life and has important social functions in Vietnam. Poultry is an important source of cash income for village families and provide a cheap source of protein for rural people.

Chickens and ducks raised in households in traditional production systems are the dominant form of poultry production in rural areas. The local chicken industry remains mainly family-run enterprises, which are concentrated at villages around towns and cities.

Scavenging and backyard chickens are common in peasant families. Due to lack of funds and land surface restrictions, the small farmers very often grow 5 to 50 chickens including layers and broilers. Kitchen waste, redundant of agricultural production such as rice bran and maize, are used as feed. Normally, free-range systems don't have houses; chickens scavenge feed in the early morning and return in the late afternoon and sleep on the perches in side pig houses. In back-yard systems, simple pens are located in the back yard or next to the inhabited houses. The pens are usually made of local materials (bamboo, wood, brick etc.). Chickens are fed commercial feed combined with local feed.

Duck meat is gaining increasing popularity among the consumers in Vietnam, thus duck rearing is developing at present. A traditional way of keeping ducks is the most common form. Both laying ducks and meat ducks are grown. Laying ducks are kept throughout the year; they are normally kept free-range and they seek feed in the arroyo and rice field by daytime and return home by night. The meat ducks, however, are kept in the field, because meat duck flocks are bigger and producers use them to collect the fallen rice during two harvest seasons (May and October). Recently, with the introduction of super-meat ducks and muscovy ducks from abroad, the industrial way of raising ducks is just begun in some areas (Personal observation).

According to T.D. Nguyen (Personal communication, 2004) some main factors are constraining the village poultry production:

Local poultry is held in every household, the majority of them are kept as free range; there is to-and-fro among closed flocks. It is easy for diseases to be introduced and it is difficult to control diseases when they occur.

In many households, people keep ducks and chickens at the same time. The birds use to live together in the backyard.

Chicken and duck pens are made of local materials (bamboo, wood, brick etc.), including those used for growing industrial broilers. Feces and urine are excreted directly to the environment. For this reason, bio-security is very difficult/impossible to practice.

Due to the small-scale poultry production, the traders have to go from one house to another to collect live birds and then sell them at the markets. Thus the control of animal movement and disease is nearly impossible.

In the markets, birds are sold alive. Vietnam dose not have a dedicated slaughtering facility neither for the industry nor for land-based poultry. All slaughtering of live poultry is done in facilities associated with households, retail markets or shops selling these birds. Under such conditions, the sanitary meat inspection is very scarce.

At present, the Vietnamese government has with a flexible policy encouraged farmers to develop poultry production in small households. The government has together with foreign donors, including DANIDA, initiated several projects aimed at improving health status and productivity of the rural animal production. Small Livestock Component, a part of The Agricultural Sector Program Support – DANIDA, has a project on improved small livestock (pigs, chicken, ducks) production and income generation among poor smallholders in some provinces in northern Vietnam.

Currently, an epidemic of High Pathogenic Avian Influenza (HPAI) is ongoing in Vietnam and other parts of Asia. The disease is considered a disaster to both veterinary and human health. Avian Influenza A (H5N1) infected both chickens and humans in Hong Kong in1997 (Anonym, 2004_a). This was the first time an avian influenza virus was demonstrated to transmit directly from birds to humans. During this outbreak, 18 people were hospitalized and 6 of them died (Class et al., 1998, Subbarao, 1998). To control the outbreak, authorities killed about 1.5 million chickens to remove the source of the virus but new genotypes of H5N1 virus continued to emerge in poultry in Hong Kong in 2000 and 2001 (Webster et al., 2002, Guan et al., 2002), and in 2003, antigenically and biologically novel H5N1 influenza virus killed one of two infected humans (Sturm-Ramirez et al., 2004). An outbreak of H7N7 avian influenza virus that caused highly pathogenic avian influenza on 225 poultry farms in Holland in 2003 was associated with conjunctivitis in 347 humans (Abbott, 2003).

In Vietnam, Avian influenza was officially declared as occurring in the country on 23rd December 2003 (Source: Animal Health Dep, 2003). The first outbreak was reported in Long An and Tien Giang province in the South of Vietnam. At the same time the disease was reported in Ha Tay province in the North Vietnam. The appearance of disease in Thai Binh province first occurred at a breeding farm on the 16th of January 2004. According to the Department of Animal Health, the disease was reported in 57/61 provinces and appeared in 1691 communes during the period 27th December 2003 to 6th February 2004. About 30 millions birds were infected (sick or dead birds) during February 2004 (Source: Animal Health Dept. 2004). It was reported that about 40 million birds, or 15 per cent of the country's stock, were killed or culled across the country, and that the poultry industry's losses were approximately 3 trillion VND (US$ 190 million) (Anonym, 2004_b).

Many investigations on the Ecology and Epidemiology of Avian Influenza have been performed. A surveillance study on Influenza A virus in Wild Birds in Northern Europe in 1999-2000 by Fouchier et al., (2003) showed that many different influenza A virus subtypes were found to circulate at the same time, in the same bird species at a single location in the Netherlands. A study of Hanson et al., (2003) on Avian Influenza virus in Minnesota Ducks during 1998-2000 determined that hemagglutinin (HA) subtype H3, H4, H6 were predominant together with H5, H7, H9.

No investigations on AI in neither commercial chickens or in village chickens had been performed in Vietnam until the outbreak in December 2003. As a consequence no information is available from Vietnam concerning seroprevalence, circulation, virulence and other characteristics concerning AI virus. In general, little information has been published concerning infectivity of AI virus following shedding to the environment and subsequent infection to other susceptible birds.

The present study aimed at determining the circulation of Avian Influenza virus in village poultry in Thai Binh province indirectly by serology and directly by virus propagation in embryonated eggs and reverse transcription-polymerase chain reaction (RT-PCR). In addition, an experiment was designed to answer the question of survival time of the virus in different aquatic environments. The studies were performed approximately one month following the last recognized outbreak in Thai Binh and were focused on elucidating basic aspects of the post epidemic epidemiology of AI in Vietnam.

The overall objective was to provide information on seroprevalence and the AIV subtype diversity in village poultry and on the resistance of H5N1 AIV to different environmental factors.

The specific objectives:

- To determine the prevalence of antibody to H3, H5, H12 virus in chickens and domestic waterfowl (duck and moscovy duck) in rural areas.

- To investigate the influence of different factors (age, species, modality of poultry production) on AI antibody prevalence in village poultry.

- To isolate the AIV subtypes present in village domestic birds.

- To test the survivability/infectivity of AIV following incubation of virus in brackish, pond and arroyo water at different temperatures.

1. Influenza

Influenza is caused by viruses from the family *Orthomyxoviridae* and is recognized as the cause of a significant number of natural infections and disease, usually of the upper respiratory tract in humans, horses, domestic pigs, and various bird species (Lvov et al., 1978; Webster et al., 1992). Domestic poultry infected with avian influenza (AI) virus commonly show symptoms ranging from asymtomatic infection to respiratory disease and drop in egg production to severe systemic disease with high mortality often up to 100% (Easterday at al., 1997). Perroncito (1878) first distinguished the disease from bacterial diseases, such as fowl cholera. Before 1981, avian influenza was known as “fowl plague”, but thereafter it has been termed highly pathogenic avian influenza (HPAI) (Anon, 2000). The Office International des Epizooties (OIE), a World Trade Organization allied group that codifies sanitary and health standards HPAI as a List A disease (Alexander, 1996_a).

Writing in 1930, Todd and Rice (1930) considered HPAI to have occurred in Austria, Switzerland, France, Belgium, The Netherland, Egypt, China, USA, Argentina and Brazil (Krohn, 1925; Mohler, 1926 and Stubbs, 1948). From the mid-1930s to 1959, there were few reports describing HPAI outbreaks appearing in the literature, but authors often stated that outbreaks were not uncommon in Africa, Asia and Eastern Europe (Anon, 2000). Since the discovery of AI virus as the cause of fowl plague in 1955, there have been 19 reported outbreaks of HPAI in domesticated poultry, mainly in chickens and turkeys, but one outbreak was reported in wild birds (i.e., common terns) (Swayne & Halvorson, 2003) (Table 1).

Economic losses from avian influenza to the poultry production have been significant in many countries. Direct losses in HPAI outbreaks have included costs for depopulation and disposal, costs due to high morbidity and mortality, quarantine and surveillance, and indemnities paid for elimination of marketing birds (Swayne & Halvorson, 2003). The HPAI H5N2 outbreaks which appeared in 449 commercial farms including more than 17 million poultry in the northeastern United States in 1983-1984 (Fichtner, 1987) were estimated by the U.S. federal government to have cost more than $ 63 million in direct eradication costs including $ 40 million in indemnities (Lasley, 1986, Fichtner, 1987). Moreover, many human cases of H5N1 virus infection were reported from Asian countries (China, Hong Kong, Vietnam) during the last 10 years. An HPAI virus (H5N1) resulted in the hospitalization of 18 people and six deaths in Hong Kong in 1997 (Mounts et al., 1999).

Table 1. The only nineteen documented outbreaks of HPAI since the discovery of AI virus as the cause of fowl plague in 1955 (Swayne & Halvorson, 2003).

AI virus	Subtype
A/ chicken/ Scotland/59	H5N1
A/tern/South Africa/6 1	H5N3
A/turkeyEngland/63	H7N3
A/turkey/Ontario/7732/66	H5N9
A/chicken/Victoria/76	H7N7
A/chicken/Germany/79	H7N7
A/turkey/England/199/79	H7N7
A/chicken/Pennsylvania/1370/83	H5N2
A/turkey/Ireland/1378/83	H5N8
A/chicken/Victoria/85	H7N7
A/turkey/England/50-92/91	H5N1
A/chicken/Victoria/92	H7N3
A/chicken/Queenland/95	H7N3
A/chicken/Puebla/8623-607/94	H5N2
A/Queretaro/14588-19/95	
A/chicken/Pakistan/447/95	H7N3
A/chicken/Hong Kong/220/97	H5N1
A/chicken/New South Wales/1651/97	H7N4
A/chicken/Italy/330/97	H5N2
A/turkey/Italy/4580/99	H7N1
A/chicken/Hong Kong/2001	H5N1

1.1. Etiology of Avian influenza

Avian influenza viruses are classified in the family Orthomyxoviridae, genus Influenzavirus A (Cox et al., 2000). Virions are typically spherical to pleomorphic but can be filamentous (Cox et al., 2000). The surface is covered by two types of glycoprotein projections (10 –14nm in length and 4 – 6 nm in diameter): 1, rod-shaped trimers of hemagglutinin (HA), and 2, mushroom-shaped tetramer of neuraminidase (NA) (Fig. 1.1) (Cox et al., 2000).

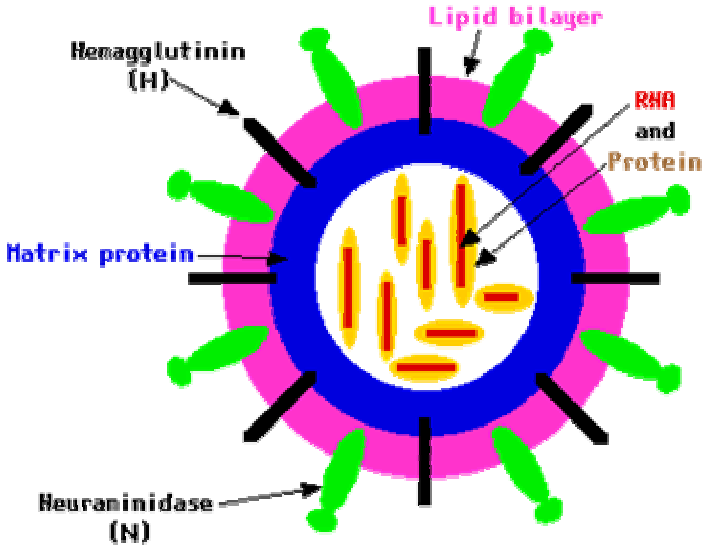


Figure 1. Surface structure of avian influenza virus with two types of glycoprotein projection (Hemagglutinin-H and Neuraminidase-N).

Influenza viruses divide into three types: A, B and C. Type A influenza viruses are further divided into subtypes based on the antigenic relationship in the surface glycoproteins haemagglutinin (HA) and neuraminidase (NA). At present 15 HA subtypes have been recognized (H1-H15) and nine NA subtypes (N1-N9). Each virus has one HA and one NA antigen, apparently in any combination. To date only viruses of H5 and H7 subtypes have been shown to cause HPAI in susceptible species, but not all H5 and H7 viruses are highly virulent.

For all influenza viruses the haemagglutinin glycoprotein is produced as a precursor, HAO, which requires post translational cleavage by host poteases before it is functional and virus particles are infectious (Rott, 1992). The HAO precursor proteins of avian influenza viruses of low virulence for poultry have a single arginine at the cleavage site and another at position –4. These viruses are limited to cleavage by host proteases such as trypsin-like enzymes and thus

restricted to replication at sites in the host where such enzymes are found, i.e. the respiratory and intestinal tracts. HPAI viruses possess multiple basic amino acids [arginine and lysine] at their HAO cleavage sites either as a result of apparent insertion or apparent substitution (Vey et al., 1992; Wood et al., 1993; Senne et al., 1996) and appear to be cleavable by a ubiquitous protease, probably one or more protein-processing subtilisin-related endoproteases of which furin is the leading candidate (Stieneke-Grober et al., 1992).

The viral genome is composed of eight segments of single-stranded, negative-sense RNA that code for 10 proteins. Their functions are shown in table 2. Eight proteins are constituents of the virus (HA, NA, NP, M1, M2, PB1, PB2, and PA), and the two nonstructural proteins (NS1 and NS2) are located in host cell cytoplasm (Lamb & Krug, 1996).

The stages of virus replication are illustrated in figure 2. AI virus HA adsorbs to host cell receptor containing sialic acid bound to glycoproteins, thus initiating receptor-mediated endocytosis. In the endosomes, low-pH-dependent fusion occur via HA-mediated fusion of viral envelop with the endosome membrane. Proteolytic cleavage of HA into HA1 and HA2 is an essential prerequisite for fusion and infectivity.

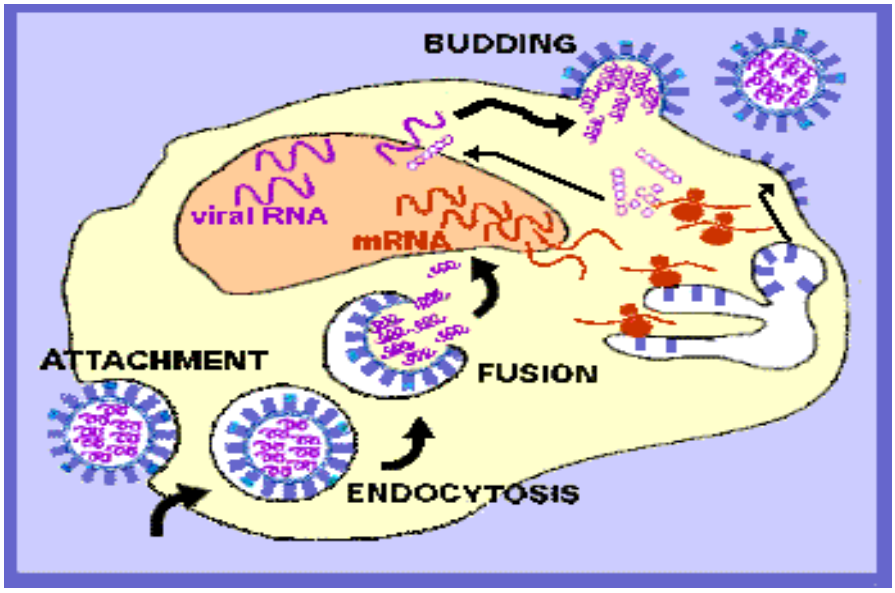


Figure 2: The stages of virus replication in host cell

The viral nucleocapsids are transported to the nucleus where viral transcriptase complex synthesizes mRNA. Transcription is initiated with 10-13 nucleotide RNA fragments generated from host heterogenous nuclear RNA via viral endonuclase activity of PB2. Six monocisttronic mRNAs are produced in the nucleus and transported to the cytoplasm for

translation into HA, NA, NP, PB1, PB2, and PA proteins. The mRNA of NS and M genesegment undergo splicing with each producing two mRNAs, which are translated into NS1, NS2, M1, and M2 proteins. The HA and NA proteins are glycosylated in the roug endoplasmic reticulum, trimmed in the Golgi and transported to the surface where they are embedded in the plasma membrane. The eight viral gene segments along with internal viral proteins (NP, PB1, PB2, PA, and M2) assemble and migrate to areas of the plasma membrane containing the integrated HA, NA, and M2 proteins. The M1 protein promotes close association with the plasma membrane and budding of the virions (Cox et al., 2000; Lamb & Krug, 1996; Easterday et al., 1997).

Table 2. Protein function in Influenzavirus A (Swayne & Halvorson., 2003).

Protein coded	**Function**
PB1	Transcriptase
PB2	Endonuclease
PA	1. Viral RNA replication. 2. Proteolytic activity
Hemagglutinin (HA)	1. Virus attachment to sialyloligosaccharide cell receptors including hemaggluinating activity. 2. Envelop fusion. 3. Antibody-mediated viral neytralization
Nucleoprotein (NP)	1. Cytoplasmic to nuclear transport of viral RNP. 2. Necessary for full length vRNA synthesis. 3. Antigen target for cytotoxic T lymphocytes
Neuraminidae (NA)	1. Cell receptor-destroying enzyme (sialic acid residues) that cause virus elution. 2. Antibody-mediated virus neutralization restricts virus spread
Matrix 1 (M1)	Most abundant protein-role in virus pudding
Matrix 2 (M2)	Ion channel
Non-structural 1 (NS1)	1. Inhibit processing of cellular mRNA. 2. Enhance of cytoplasmic translation of viral mRNA. 3. Possible inhibition of interferon pathways
Non-structural 2 (NS2)	Nuclear export of viral RNA

Because of genetic instability of influenza viruses, viruses with new combination of genes can and do develop. All type A influenza viruses, including those that regularly cause seasonal epidemics of influenza in humans, are genetically labile and well adapted to elude host defenses (Swayne & Havorson, 2003). Influenza viruses lack mechanisms for the

"proofreading" and repair of errors that occur during replication. As a result of these uncorrected errors, the genetic composition of the viruses' changes as they replicate in humans and animals, and the existing strain is replicate with a new antigenic variant. These constant, permanent and usually small changes in the antigenic composition of influenza A viruses are known as antigenic "drift". Influenza viruses have a second characteristic of great public health concern: influenza A viruses, including subtypes from different species, can swap or "reassort" genetic materials and merge. This reassortment process, known as antigenic "shift", results in a novel subtype different from both parent viruses. As population will have no immunity to the new subtype, and as no existing vaccines can confer protection, antigenic shift has historically resulted in highly lethal pandemics. For this to happen, the novel subtype needs to have genes from human influenza viruses that make it readily transmissible from person to person for a sustainable period. Condition favourable for the emergence of antigenic shift have long been thought to involve humans living in close proximity to domestic poultry and pig. Because pigs are susceptible to infection with both avian mammalian viruses, including human strains, they can serve as a "mixing vessel" for the scrambling of genetic material from human and avian viruses, resulting in the emergence of the novel subtype. Recently event, however, have identified a second possible mechanism. Evidence is accumulating that, for at least some of the 15 avian influenza virus subtypes circulating in birds populations, humans themselves can serve as the "mixing vessel" (Liu et al., & Swayne & Halverson, 2003)

Avian influenza viruses are relatively unstable in the environment. Physical factors such as heat, extremes of pH, nonisotonic condition, and dryness can inactivate AI virus (Swayne & Halvorson, 2003). In the laboratory, AI virus can maintain infectivity in chorioallantoic fluid for several weeks at 4ºC and long-term storage should be at -70ºC.

Influenza viruses are protected by organic material such as nasal secretions or feces, which increase resistance to physical and chemical inactivation (Easterday et al., 1997). Cool and moist conditions favor long survival of AI viruses in the environment. AI viruses have been viable in liquid manure for 105 days in the winter and in feces for 30-35 days at 4ºC and for 7 days at 20ºC (Beard at al., 1984; Fichtner, 1987 and Webter at al., 1978).

In the present study indicated that in aquatic environment the AI virus lost its infectivity after 6 hours at 37ºC and maintained its infectivity for at least 4 days at temperatures up to 25ºC (Manuscript I).

1.2 Epidemiology of avian influenza

1.2.1. Hosts

Avian influenza viruses have a worldwide distribution; virus isolations have been reported in Africa, Asia, Australia, Europe, and South America (Easterday et al., 1997, Morgan & Weatbury, 1981; Swayne & Suarrez, 2000). Viruses have been shown to infect a great variety of birds including free-living birds, captive caged birds, domestic ducks, chickens, turkeys and other domestic poultry (Lvov, 1978; Hinshaw et al., 1981; Alexander, 2000). Viruses have been isolated from species of free-living birds covering all the major families of birds. However, the frequency of isolation and variations in subtypes seen in ducks and geese has overshadowed those from other species. It seems likely that the viruses are perpetuated in free-living birds, particularly migratory waterfowl (Hinshaw et al., 1980). Shortridge (1999) found that live poultry markets (LPM) often are infected with AI virus with high rates in both developed and developing countries. Surveys of poultry in LPM of Hong Kong, New York, and other large cities has indicated that mildly pathogenic avian influenza (MPAI) viruses have become endemic in these agricultural systems. A recent study of domestic ducks infected with several 2004 H5N1 viruses found that the quantities of virus excreted by healthy-looking ducks approach those excreted by visibly affected chickens, so domestic ducks might be acting as the a “silent” reservoir for the H5N1, which is highly pathogenic for chickens (Anon, 2004c).

1.2.2. Transmission

Some studies on the transmissibility of influenza viruses found that the transmission from bird to bird is extremely complex, and depends on the strain of virus, the species of bird, and environmental factors (Narayan et al., 1969; Alexander et al., 1978, 1986; Westbury et al., 1979, 1981). AI virus is excreted from the nares, conjunctiva, and cloaca of infected birds into the environment because of virus replication in the respiratory, intestinal, renal, and/or reproductive organs (Swayne & Halvorson, 2003). The ability of virus to spread must be related to the amount of virus excreted by the respiratory and intestinal tract. Because the highly pathogenic viruses cause extremely rapid deaths in birds, it is possible that birds release little virus during the course of such infection. Thus, virulent viruses have tended to show poorer transmission from infected birds to susceptible chickens and turkeys than viruses of low pathogenicity in both nature and experimental infection (Anon, 2000).

The virus is transmitted by direct contact between infected and susceptible birds or indirect contact through aerosol droplets or exposure to virus-contaminated fomites (Easterday et al., 1997). A study of Hinshaw et al. (1980) found that the perpetuation of influenza viruses in Canadian free-living waterfowl was related to the passage of virus from adult to juvenile birds on lakes where the birds congregated before migration. Infected ducks may contaminate lakes or ponds with amounts of virus up to $10^{8,7}$ mean egg infectious doses per g of feces (Webster

et al., 1978). Hinshaw et al. (1979) have isolated AIV from untreated lake water where large numbers of waterfowl are found.

Figure 3.The poultry movment and Live poultry market in Vietnam (Photo, Dung. 2004)

Observation on the spread of AI in Vietnam in 2004 revealed that the disease emerged and spread widely in the provinces with a high population of ducks such as Long An and Tien Giang province in the South of Vietnam. Keeping many species of domestic birds in the same areas and the difficulty of controlling bird movement even with birds infected with AI were main complication factors in the outbreaks in Vietnam during 2004 (Own observation).

1.3. Prevalence of avian influenza:

Surveillance of Hong Kong poultry markets in December 1997 indicated H5N1 infections were widespread, especially in chickens (19.5% isolation rate) but also in ducks (2.4%) and geese (2.5%) (Shortridge, 1997). An isolation rate of 2.4% for H5 viruses, 0.9% for H9, and 2.7% for other AI viruses was also obtained from this survey.

A study on the presence of AI virus in free flying, nonmigrator ducks on the Eastern Shore of Maryland in the United States in 1998 showed that virus could only be recovered from ducks during a short period between mid-July and the end of August. HA subtypes H2, H3, H6, H9 and H12 were recovered from 13.9% of 209 cloacal swabs collected (Slemons et al., 2003).

Surveillance and diagnostic samples (tracheal and cloacal) from 295 flocks (commercial layer, pullet, and broiler) were tested during a nonpathogenic H7N2 avian influenza outbreak in Pennsylvania, and 15 flocks were positive with both the Directigen Flu A and virus isolation, four flocks were negative with the Directigen Flu A but positive with virus isolation, and 276 were negative with both tests. No flocks were found to be positive with the Directigen Flu A test and negative with virus isolation (Davison et al., 2003).

Surveillance on live poultry markets in Nanchang, China during 2000 showed that influenza viruses were detected from 1% of in total 6360 samples. The highest rate of virus isolation was from ducks and chickens with an isolation rate of 1.2% (30/2550) and 1.3% (17/1360), respectively. The remaining viruses were isolated from 0.8% (10/1260) of quail samples and 0.5% of pigeon samples (6/1190) (Liu et al., 2003).

Sampling of fecal trays from live poultry market in Hong Kong during 1999 showed that the rate of H9N2 virus isolation alone was 5.2 % (Guan et al., 2000).

In a survey in October 1999, 10.6% of all Mallard ducks in ducks trap Bakkerswaal in Lekkerkerk, Netherlands, were positive for influenza A virus, and in the second week of August 1999, 60% (6/10) black headed gull in Ottenby, Sweden, were positive (Fourchier et al., 2003).

A survey on circulation of influenza viruses in wild waterfowl wintering in Italy during 1993-99 showed that the overall percentages of recaptured ducks that showed in-creased antibody levels suggestive of recent infection for influenza A virus were 25.6% in the mallards and 11.1% in the other dabbling ducks. No positive was found in recaptured diving ducks (De Marco et al., 2003).

A study on avian influenza virus in Minnesota Ducks during 1998-2000 found that AIV were detected during all three years with 154 AIVs isolated from 1423 (10.8%) sampled ducks. Prevalence of infection in mallards was 18% and in northern pintails 2.9%. The prevalence of AIV, which was detected in juvenile's mallards (16.6%), was higher than in adults (2%). Viruses representative of the HA subtypes 2,3,4,5,6,7,9,10,11,12 were isolated. Viruses in the H5, H7, and H9 subtypes, which are associated with high-pathogenicity influenza in poultry or recent infections in humans, were uncommon (Hanson et al., 2003).

In the present study, only one H12 AI virus was isolated from 587 cloacal swabs birds in local poultry of Thai binh province in Vietnam. However antibodies against H5, H3 and H12 viruses were found with high prevalence and the prevalence of positive H5 sera in ducks (77.63%) was significantly higher than in chickens (5.82%) (Manuscript I)

1.4. Pathogenesis:

Initially, avian influenza viruses enter the host by inhalation or digestion of infectious LPAI or HPAI virions. Viruses easily affect poultry by this route due to trypsin-like enzymes in the respiratory and intestinal epithelial cells, which allow cleavage of the surface haemagglutinin, and thereby promote an initial replication in the respiratory and/or intestinal tract. Thus AI viruses are commonly released in nasal exudates and feces. In poultry, the nasal cavity is a major site of initial replication (Swayne & Halverson, 2003).

The HPAI virions invade the sub-mucosa and subsequently the capillaries. The virus replicates within endothelial cells and spreads via the vascular or lymphatic systems to effect and replicate in the variety of cell types of visceral organs including brain and skin. Alternatively, the virus may become systemic before having extensive replication in vascular endothelial cells. The presence of a haemagglutinin proteolytic cleavage site that can be cut by ubiquitous furin-like cellular enzymes is responsible for this pantropic replication. Clinical signs and death are due to multiple organs failure. Damage caused by AI viruses is the result of one of three processes: Direct virus replication in cell, tissues, and organs; Indirect effects from production of cellular mediators such as cytokines; and finally ischemia from vascular thrombosis (Swayne & Halverson, 2003).

For LPAI viruses, the replication of viruses usually is limited to the respiratory and intestinal tract. Illness or death is most often from respiratory damage, especially if a companied by secondary bacterial infections. Occasionally, the LPAI viruses spread systemically, replicating and causing damage in kidney tubules, pancreatic acinar epithelium, and other organs with epithelial cells having trypsin-like enzymes (Swayne & Halverson, 2003).

1.5. Clinical signs

Low pathogenic avian influenza (LPAI): Most infections by LPAI viruses in wild birds produce no clinical signs. However, in experimental studies in mallard ducks, LPAI virus infections suppressed T-cell function and produced a one-week depression in egg production (Takizawa et al., 1995; Toth & Norcross, 1981). In domestic poultry (chickens and turkey), clinical signs reflect abnormalities in the respiratory, digestive, urinary, and reproductive organs. The most frequent signs represent infection of the respiratory tract and include mild to severe respiratory signs such as coughing, sneezing, rales, rattles, and excessive lacrimation. Increased broodiness and decreased egg production may be seen in hen from layer and breeder flocks. The clinical signs of huddling, ruffled feathers, depression, decreased activity; decreased feed and water consumption and occasionally diarrhea will be exhibited in domestic poultry (Swayne & Halvorson, 2003).

Alexander and Spackman (1981) reported that an LPAI infection in a turkey laying flock resulted in only transient mild respiratory signs and 2% white-shelled eggs. Other LPAI outbreaks occurring in turkeys at about the same time produced 20-40% drops in egg production and respiratory disease with low but significant mortality. At the other extreme, infections with LPAI viruses may be associated with severe disease and with high mortality. In outbreaks in chickens in Alabama in 1975 with a LPAI virus of subtype H4N8 up to 69% mortality was recorded in infected flocks (Johnson et al., 1977). In 1995 major outbreaks caused by LPAI viruses of subtype H7N3 affected turkeys in Utah USA and was associated with significant mortality especially in young birds, with about 40% mortality in 0- to 4-week-old birds (Halvorson et al., 1998). During the LPAI H7N1 infections in Italy in 1999, turkeys were particularly affected. In turkey breeders a milder form of the same clinical condition was observed that consisted of exhibited rales, coughing and swelling of the infraorbital sinuses and a febrile condition associated with loss of appetite. Egg production dropped by 30% to 80% during the acute phase, but partially recovered to subnormal levels within three weeks from the onset of the disease. Mortality rates ranged from 5 to 20% (Capua et al., 2000).

Highly pathogenic avian influenza (HPAI): Few clinical signs have been seen in wild birds and domestic ducks due to HPAI viruses either because they replicate poorly or replicate to a limited degree (Swayne & Halvorson, 2003). The one exception has been the 1996 H5N3 HPAI outbreak in common terns in South Africa where birds died suddenly without any other clinical signs (Becker, 1996).

In domestic chickens, turkeys, and related galliformes, clinical signs reflect virus replication and damage to multiple visceral organs, cardiovascular and nervous systems. However, clinical manifestations vary depending on the extent of damage to specific organs and tissues. Often the first signs are sudden onset of high mortality, which may approach 100% within a few days. Clinical signs, which are commonly seen, include cessation of egg laying, respiratory signs, rales, excessive lacrimation, sinusitis, oedema of the head and face, subcutaneous haemorrhage with cyanosis of skin, particularly of the head and wattles, and diarrhea and occasionally neurological signs may be present. Usually, the signs are most marked in birds that take some time to die and not all clinical signs are present in each bird.

In the current epidemic in Vietnam, signs have been dominated by sudden death with high mortality up to 100% and respiratory signs such as sneezing, rattles and excessive lacrimation (Own obsevation).

1.6. Gross lesions

Gross lesions are extremely variable regarding their location and severity, depending greatly on the host species, pathogenicity of the infecting virus, and presence of secondary pathogens.

LPAI form: In the less acute form, and in mature birds, significant gross lesions are frequently observed. They may consist of subcutaneous edema of the head and neck area. Fluid may exit the nares and oral cavity as the bird is positioned for postmortem examination. The conjunctivae are severely congested occasionally with petechiation. Lesions in the respiratory tract are most frequently seen. It is characterized as catarrhal, fibrinous, serofibrinous, mucopurulent, or fibropurulent inflammation. The tracheal mucosa can be edematous with congestion and occasionally haemorrhage. Tracheal exudates may vary from serous to caseous, with occasional occlusion of airways and resulting asphyxiation. Fibrinous to fibrinopurulent airsacculitis may be observed (Swayne & Halverson, 2003).

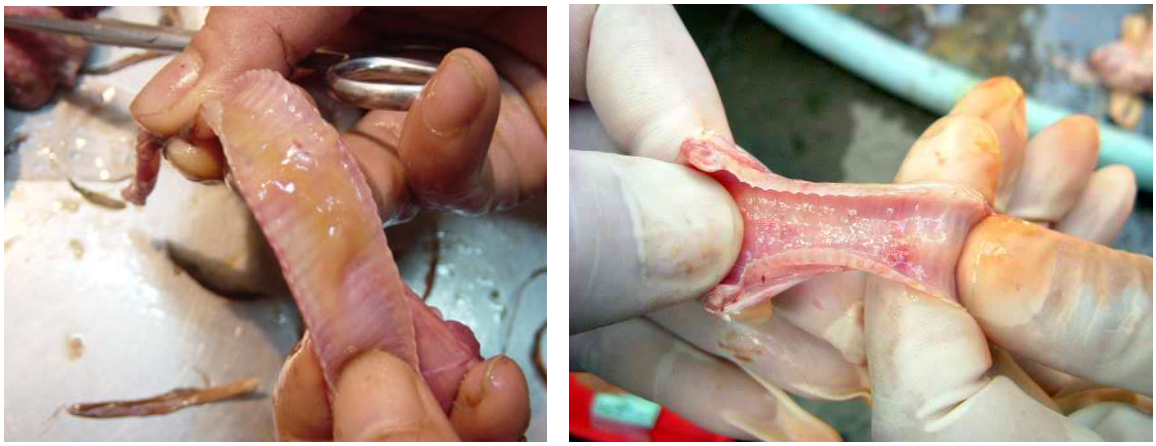


Figure 4. Gross lesions associated with fibropurulent inflammation, mucopurulent and haemorrhage in trachea (Photo, Dung 2004)

When the bird is opened, pinpoint petechial hemorrhages are frequently observed on the inside of the keel as it is bent back. Very small petechia may cover the abdominal fat, serosal surfaces, and peritoneum, which appears as if it were finely splattered with red paint. Kidneys are severely congested and may occasionally be grossly plugged with white urate deposits in the tubules (Beard. 1998).

In layers, the ovary may be hemorrhagic or degenerated with darkened areas of necrosis. The peritoneal cavity is frequently filled with yolk from ruptured ova, causing severe airsacculitis and peritonitis in birds that survive for 7 to 10 days (Beard. 1998).

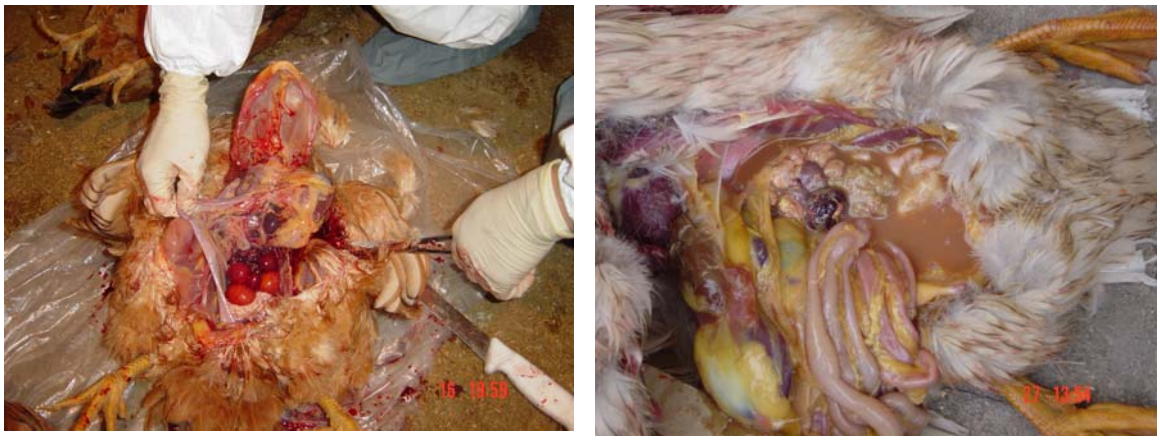
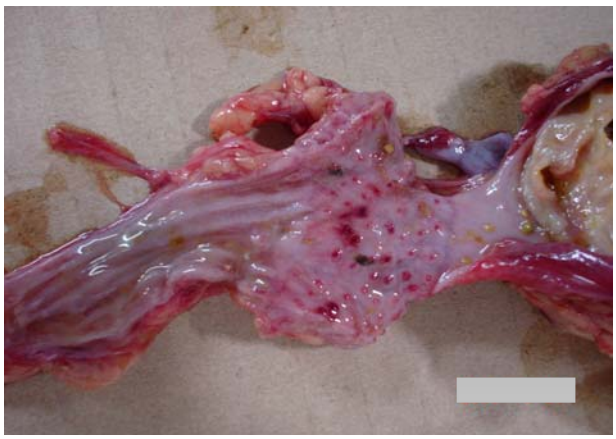


Figure 5. Petechial hemorrhages in epicardial fat and ruptured ova, peritonitis (Photo, Dung 2004)

Petechial hemorrhages may be present on the mucosal surface of the proventriculus — particularly at the juncture with the gizzard. The lining of the gizzard peels easily and frequently reveals hemorrhages and erosions underneath. The intestinal muscosa may have hemorrhagic areas especially in the lymphoid foci such as the cecal tonsils. The gross lesions are not distinctly different from those observed with velogenic viscerotropic Newcastle disease (VVND). The lesions in turkeys and domestic ducks are similar to those in chickens but may not be as marked (Beard. 1998).

Figure 6. Gross lesions associated with petechial hemorrhages around the ducts of the proventricular glandular region (Photo, Dung 2004)



HPAI form: Birds that die with the peracute disease and young birds may not have significant gross lesions other than severe congestion of the musculature and dehydration. In chickens, swelling of the head, face, upper neck, and feet are common as the result of subcutaneous edema and may be companied by petechial to ecchymotic hemorrhage *(Figure 7)*. Necrotic foci, hemorrhage and cyanosis of the non-feathered skin are common, especially wattle and combs. Lesions in visceral organs vary with virus strain but most consistently are represented

by hemorrhage on serosal or mucosal surfaces and foci of necrosis within parenchyma of visceral organs. Especially prominent are hemorrhages on the epicardium, in pectoral muscles, and mucosa of the proventriculus and ventriculus (Swayne & Halvorson., 2003).

With most HPAI viruses, necrotic foci are common in pancreas, spleen and heart, and occasionally in liver and kidney. The kidney lesions may be accompanied by urate deposits. Lungs have focal ventral to diffuse interstitial pneumonia with edema. The lungs can be congested or hemorrhagic. The cloacal bursa and thymus are usually atrophic (Swayne & Halvorson., 2003).

Figure 7. Necorsis and hemorrhage of comb and wattle, and subcutaneous hemorrhages of leg shanks (Photo, Dung 2004)

In the AI outbreaks in Thai Binh province in Vietnam, gross lesions in the respiratory and intestinal tracts were common. Fibropurulent inflammation, mucopurulent and haemorrhage in trachea were observed. Some lesions in visceral organs such as petechial hemorrhage cover the abdominal fat, serosal surfaces and peritoneum; petechial haemorrhages in proventriculus, and haemorrhage in ovary were frequently observed in individual birds (Own observation).

1.7. Diagnosis

Field diagnosis: Highly pathogenic avian influenza may be suspected within any flock where sudden deaths follow severe depression, inappetence, and a drastic decline in egg production. The presence of facial edema, swollen and cyanotic combs and wattles, and petechial hemorrhages on internal membrane surfaces increases the likelihood that the disease is HPAI. However, an absolute diagnosis is dependent upon the isolation and identification of the causative virus. Commercially available type A influenza antigen-capture enzyme linked immunosorbent assay kits designed for uses in human influenza have recently shown promise as a possible rapid diagnostic test for poultry.

Specimens for the laboratory: Specimens sent to the laboratory should be accompanied by a history of clinical signs and gross lesions, including any information on recent additions to the flock. Diagnosis depends upon the isolation and identification of the virus from tracheal or

cloacal swabs, feces, or from internal organs (Beard, 1989). Specimens should be collected from several birds. It is not unusual for many of the submitted specimens to fail to yield virus. Swabs are the most convenient way to transfer AI virus from tissues or secretions of the suspect bird to brain and heart infusion broth or other cell culture maintenance medium containing high levels of antibiotics. Dry swabs should be inserted deeply to ensure ample epithelial tissue. Trachea, lung, spleen, cloaca, and brain should be sampled. If large numbers of dead or live birds are to be sampled, cloacal swabs from up to five birds can be pooled in the same tube of broth. An alternative technique is to place 0.5 cm^3 of each tissue into the broth. Blood for serum should be collected from several birds. If the specimens can be delivered to a laboratory within 24 hours, they should be placed on ice. If delivery will take longer, quick-freeze the specimens and do not allow them to thaw during transit. If the samples must be held for additional time, storage at - 70ºC is recommended. Before testing for virus, tissues should be ground as a 5 – 10 % suspension in the transport medium and clarified by low-sped centrifuge.

Virus isolation: Centers for Disease Control. (1982), Easterday et al. (1997), and Swayne et al. (1998) have described methods for isolation and identification of influenza viruses in detail. Nine to 11-day-old embryonated chicken eggs are inoculated via the allantoic cavity with approximately 0.2 ml of swab or tissue specimens. The death of inoculated embryos within 24 hours after inoculation usually results from bacterial contamination or inoculation injury. Avian influenza virus will usually kill embryos within 48-72 hours. After 72 hours, or at death, the egg should be removed from incubator, chilled and allantoic fluids should be collected. If the virus isolated is identified as a Type A influenza virus, through the AGP or ELISA tests, it is then tested using a battery of specific antigens to identify its serologic identity (HA and NA type).

Generally, if virus is present in a sample, there will be sufficient growth in the first passage to result in haemagglutination, and repeated passage is unnecessary. Repeated passage of samples increases the risk of cross-contamination in the laboratory. Long-term storage of viruses should be done at -70ºC.

Virus identification: Standardized methods for testing the egg fluids for the presence of hemagglutinating activity using chicken erythrocytes by macro- or micro-techniques are employed (Centers for Disease Control, 1982; Easterday et al., 1997, and Swayne et al., 1998). Allantoic fluid positive for hemagglutination is useful for virus identification.

The allantoic fluid positive for hemagglutination should be tested in HI assays against Newcastle disease and other antiserum to determine whether the hemagglutinating activity

detected in the allantoic fluid is due to influenza virus or other hemagglutinating viruses. The type-specific NP (nucleoprotein) or matrix protein may be detected by the double immunodiffusion test (Beard, 1970; Dowdle & Schild, 1975) or the single-radial-hemolysis test (Dowdle & Schild, 1975).

The NA subtype is identified by a micro-NI assay with antisera prepared against the nine known NAs (Palmer et al., 1975; Swane et al., 1998; Van Deusen et al., 1983). The HA is identified in the HI test (Swane et al., 1998) using a panel of antisera prepared against the 15 distinct HAs. Typing id facilitated by using antisera against the isolated HA or against reassortant viruses with relevant NAs; this help avoid steric inhibition due to antibody against the NA (Kendal, 1982).

Serology: ELISA assay have been developed to detect antibodies to avian influenza virses (Abraham et al., 1986; Beck & Swayne, 1998, Fatunmbi et al., 1989; Meulemans et al., 1987; Shafer et al., 1998; Snyder et al., 1985 and Zhou et al., 1998)

Differential diagnosis: Highly pathogenic avian influenza is easily confused with VVND, because the disease signs and postmortem lesions are similar, and may also be confused with airway infection such as avian pnemovirus and other paramyxoviruses, infectious laryngotracheitis, infectious bronchitis, and acute bacterial diseases such as fowl cholera and Escherichia coli infection (Swayne & Halvorson, 2003; Beard, 1989). However, in an area where AI is prevalent, such as during an outbreak, flock history, signs, and gross lesions can make sound presumptive diagnoses.

2. Prevention and control of avian influenza

Methods for the prevention and control of avian influenza virus infection focus on the preventing the initial introduction of the virus and controlling spread if it is introduced.

Prevention: The principle for the prevention of infection of poultry with influenza viruses is the separation of susceptible birds from infected birds and their secretions and excretions. Biosecurity is the utilization of best management practices to reduce the risk of introducing avian influenza virus in a poultry house, farm or operation either for initial case or secondary case in an ongoing outbreak or preventing movement of avian influenza viruses from infected birds to susceptible birds. Normally, these practices are focusing on preventing the movement of infected poultry and their by-products such as manure, urine; preventing the movement of contaminated equipments, clothing, and shoes off of farms with infected birds. Depopulation

and cleaning & disinfection of pens, equipments and entire infected areas are commonly applied to any outbreak. A control procedure were implemented in Netherlands 2003 where all poultry was slaughtered in a 1 km zone around the sources of infection, poultry was banned moving in a zone at lest 10 km around the sources of infection and screening of farms in a radius of 3 km around the source of the infection was initiated (Cees Verman, 2003)

Control: Because avian influenza virus is excreted from both the respiratory and digestive tracts, and contaminated poultry manure and exudates are considered as a source of transmission between flocks, all methods for controlling the spread of avian influenza are based on preventing contamination and controlling the movement of peoples and equipment (Swayne & Halvorson, 2003). With the realization that there is a reservoir of influenza virus in wild waterfowl, every effort must be made to prevent direct or indirect contact between domestic poultry and wild waterfowl. Persons handling wild game (especially waterfowl) must change clothes completely and bathe prior to entering poultry houses. All buildings should be cleaned and disinfected after an infected flock is removed. The poultry litter or manure should be composted before application to cultivated lands. A package of control measures including stamping out infected herds, pre-emptive culling of surrounding flocks, movement bans, screening and tracing and hygienic measures were applied to control HPAIV H7N7outbreak in The Netherlands 2003 (Stegeman, 2003). Specific strategies used in USA for HPIV prevention, control and eradication during the 20^{th} century were quarantine of infected flocks, depopulation of flocks and disposal of carcasses, cleaning and disinfection (C&D) of equipment and farms, diagnostics and surveillance testing (1983-84) and indemnities paid for destruction of poultry (1983- 84) (Swayne & Akey, 2003).

Vaccination: The H antigen appears to be the most important antigen and birds are susceptible to infection with influenza viruses belonging to any of the 15 HA subtypes. It is not practical possible to use preventive vaccination against all possible subtypes. Thus, it is very difficult to select viruses for vaccine production. However, when a certain subtype of AI virus is identified after the start of an outbreak, a suitable vaccine may be a useful tool. Inactivated influenza virus vaccines have been used in Minnesota, other states of USA and Italy (Alexander, 1996_b). To eradicate the LPAI H5N2 outbreak in Mexico, avian influenza surveillance of commercial farms and backyard poultry were carried out from 1995 to 2001. All backyard poultry positive for AI by serology and/or viral isolation have been slaughtered and destroyed. Over 1 billion doses of inactivated-emulsified vaccine against avian influenza have been authorized during 1995 – 2001, and from May 1998 until December 2001, 459 million of doses of recombinant pox-AI vaccine have been authorized. Both vaccines have proved to be a great help to control the disease (Villarreal-Chavez & Rivera-Cruz, 2003).

In an LPAI H7N1 outbreak in Italy (2000-01), a total of 11.423.000 birds including meat-type turkey, laying hens and capons were vaccinated on 315 farms. Serum samples obtained from 16.072 sentinel birds in vaccinated flocks were negative for anti-AI antibodies.
However, a debate has been going on about the use of vaccination for avian influenza control. Some suggests that vaccination will increase the risk of virus reassortment. Beard (1998) stated that Inactivated oil-emulsion vaccines, although fairly expensive, have been demonstrated to be effective in reducing mortality, preventing disease, or both, in chickens and turkeys (7). These vaccines may not, however, prevent infection in some individual birds, which go on to shed virulent virus. More economical viable vaccines prepared using naturally virulent or attenuated strains have the disadvantage of the possible creation of reassortant influenza viruses with unpredictable characteristics. These reassortants could result when a single host bird is simultaneously infected with both the vaccine strain and another AI virus. Due to the segmented nature of the influenza virus genome, a reassortment of genetic material can readily occur, creating new influenza viruses.

3. Results

3.1. Field results

Some characteristics of Thai Binh province: Thai Binh is an agricultural province, located in the Red River delta area with the typical character of flat land. Thai Binh covers an area of 154,224 ha, in which cultivated fields accounts for 96,567 ha. The population of Thai Binh consists of 1,8 million people, 90 % of which are dependent upon rice cultivation. The total rice production accounts for more than 1 million tons per year; it was the province with the highest rice yield in the country last year (12 – 13 quintal/ha).

Thai Binh has a sound mixture of animal production and cultivation. Land previously used for rice production is now replaced by poultry, pig or dairy production farm. Therefore, the developing rate of animal production has increased, and the livestock production accounts for approximate 30% of the total agricultural production. In 2003, the population of cattle was 50 thousand heads (44% increase in comparison with 2002); the pig population is 900 thousand heads (14% increase in comparison with 2002), including a high population of sows (202,700 heads). The poultry population has also increased during the last five years with a population of 5 million in 2000 and 7,5 million in 2004, including 6 million chickens and 1.5 million ducks and moscovy ducks. The population of quails, geese, pigeons and other birds is very small.

Figure 8. Backyard and scavenging poultry systems in Vietnam (Photo, Dung & Phuong. 2004)

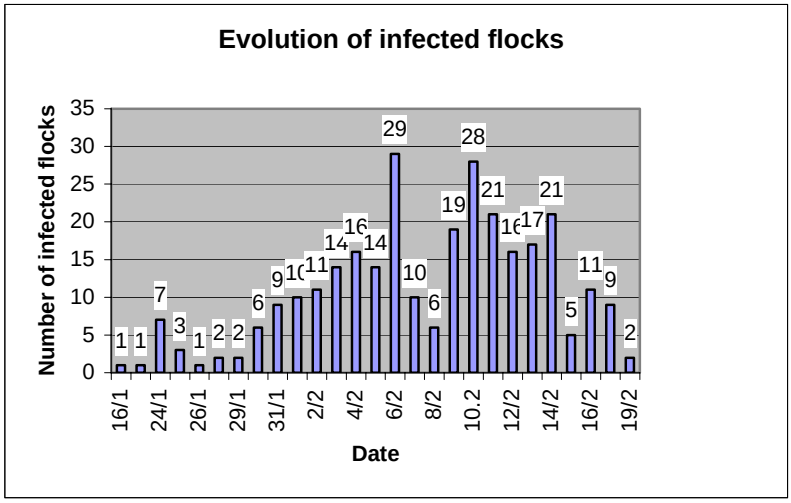
By early 2004, there were 54 chicken farms with flock sizes of 1,000 to 5,000 birds and 2,902 chicken farms with flock sizes under1000 birds. The duck farms are inferior in numbers, there were only 11 duck farms with a holding capacity of 1,000 to 5,000 ducks or moscovy ducks and 855 farms keep under1000 ducks (Thaibinh SDAH data. 2004). Most farms grow broilers, there are few breeding farms that provide day old chicks to the household sector and the latter grow them as broilers. However, scavenging and backyard poultry are dominant forms of all poultry production in Thai Binh. About 75% of the poultry population is kept in households.

Avian influenza monitoring in Thai Binh province: AI first occurred on 16th of January 2004 on a breeding farm of the Livestock Breeding Company of Thai Binh province that is located in Thai Binh town. Sudden death with high mortality appeared in chicken layer flocks and more than 500 layers died during one night (16th of January). After that, some outbreaks were reported from chickens; ducks and muscovy ducks flocks that were located around town and subsequently from numerous flocks from other districts.

The disease mainly occurred the first half of February 2004. During a short time numerous flocks was reported infected with avian influenza (Figure 9). High morbidity and mortality was observed, there were 29 infected flocks identified on 16th of February, and 12748 birds were dead or have been culled.

After one month, the disease quickly spread in 8 districts including 111 villages. In total 272 infected flocks were reported of which there were only 31 duck and muscovy duck flocks, the remaining were chicken flocks. About 1,400,000 birds were killed and destroyed, and the losses was estimated to be 34 billion VND ($266 thousands) (Thai Binh People's Committee Report)

Figure 9. Evolution of avian influenza infected flocks in Thai Binh province from 16/1 to 16/2/2004



Postmortem and diagnosis: The disease in Thai Binh province was diagnosed as being caused by H5N1HPAI. All domestic poultry species were reported infected. Ducks and muscovy ducks were mainly affected in the beginning of the outbreaks. The most frequent signs observed were sudden death, high morbidity and mortality. The clinical signs included ruffled feathers, huddling, depression, and sometime diarrhea. Birds died without any obvious gross lesions except sever congestion of the musculature and dehydration. Occasionally some typically gross lesion of avian influenza were found such as swelling and petechial to ecchymotic haemorrhage of the head, face, upper neck, and feet; haemorrhage and cyanosis of the non-feathered skin. During postmortem of death birds, in some cases, haemorrhages on the epicardium, in the pectoral muscles, and mucosa of the proventriculus and ventriculus were observed. Ruptured ova, severe airsacculitis and peritonitis have occasionally been seen in layer birds.

Avian influenza prevention and control: Specimens were collected from the first outbreak and send to National Veterinary Diagnosis and National Institute of Veterinary Research for diagnosis. By isolation of the virus in embryonated chicken eggs and HA and HI tests and the RT-PCR test, H5N1 avian influenza virus were detected, Due to that the outbreak number increased rapidly, a new definition of an outbreak was formulated. A new outbreak was defined by the epizoothiological principles of 10% death birds in 2 days or observation of hemorrhagic lesions in the internal organs.

Avian Influenza Control Commissions of provinces, districts and communes were established. The new cases and other issues in relation to AI were reported to the Control Commission on a daily basis.

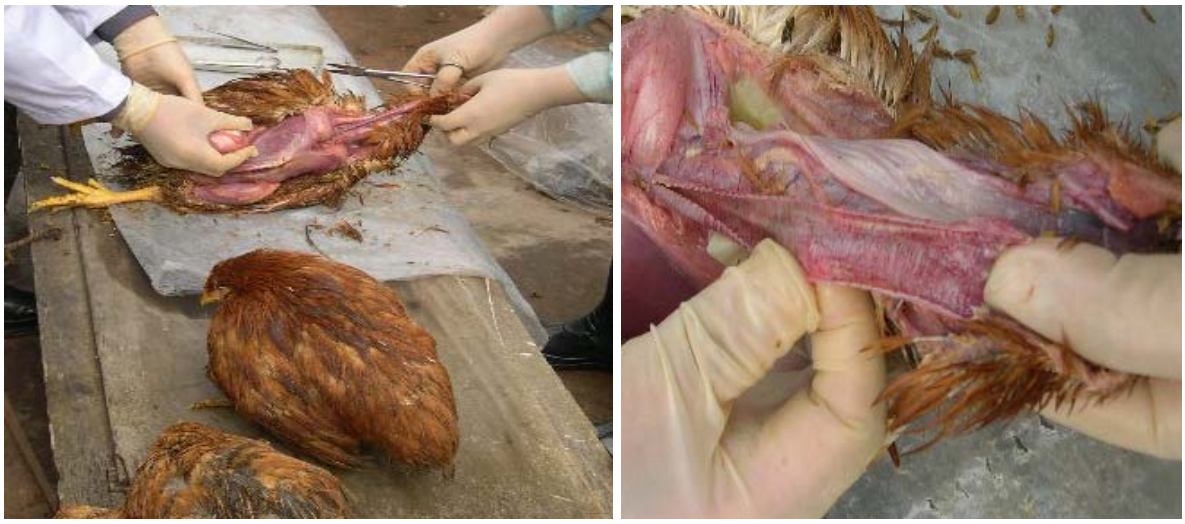


Figure 10. Diagnosis based on hemorrhagic lesions in the internal organs (Photo, Phuong. 2004).

A strict ban was enforced on trading, moving of animals, slaughtering, and consumption of poultry and poultry products at farms, households, markets and restaurants. Checkpoints were set up to prevent the moving of birds in and out of the provinces. All infected birds were killed and buried.

Disinfectants were supported free from the Government together with foreign donors including the Small Livestock Component – SAPS (DANIDA). Disinfectants were widely used not only in affected farm but also in non-affected ones, especially in breeding farms as a preventive measure to protect the breeding stock.

Vaccines were not used to control the outbreak. In summary, the main method of control of the disease was detection and stamping out.

3.2. Seroprevalence and virus isolation

In the present study, a survey on antibodies against H5 AI virus was subsequently carried out in five communes from three typical areas of Thai Binh province. In total, 587 blood and cloacal samples were obtained from 379 chickens, 132 muscovy ducks and 76 ducks for the detection of H5, H3 and H12 antibodies. The results showed that all three types of antibodies were observed from chickens, ducks and muscovy ducks with titres ranging from *1log2* to *7log2*. The H5 antibody levels in chickens were frequently higher than the two others (53% of samples have H5 tire < 3*log*2) but the prevalence of H5 antibody in ducks and moscovy ducks (77.6%, 24.1%) was significantly higher than in chickens (5.8%) ($p = 0.0001$).

The H5 antibody prevalence was analysed in relation to different factors including poultry species, bird age, flock type, geographical area, and production system (table 1).

Regarding the influence of bird age to H5 antibody prevalence, there was no significant difference among adult and layer chickens but none of the chickens less than 2 month old were seropositive. The prevalence of H5 antibody in layer ducks (87.5%) was significantly higher than in young (< 2 month old) and adult (> 2 month old) ducks (25%, 25%). In contrast, none of the fifteen layer muscovy ducks tested was seropositive for H5 but a high proportion of the young (75%) and adult birds (21.7%) was seropositive for H5.

The seroprevalence of H5 antibody among the three species from single and mixed flocks did not differ significantly in this study (p-value was bigger than 0.05). The different geographical areas did not differ concerning the antibody prevalence in the different poultry species.

However in muscovy ducks, the prevalence of positive H5 antisera of muscovy ducks kept in coastline areas were significantly higher than in muscovy ducks kept at riverside and interior areas.

The proportion of chickens having positive H5 antisera were significantly higher in chickens kept in indoor systems than in scavenging and backyard systems. A significant difference in antibody prevalence among ducks and muscovy ducks kept in the three types of production systems was not observed.

The seroprevalence of H5, H3 and H12 antibodies were also investigated in this study. The presence of these three antibodies could be in the same flock or in the same bird. The prevalence of H5 antibody was very high in ducks (77.63%); it was lower in moscovy duck (24.06%) and lowest in chickens (5.82%). In contrast, the seroprevalence of H3 and H12 antisera in chickens (36.2%, 30.6%) was higher than in ducks (10.5%, 14.4%) and moscovy duck (17.2%, 5.2%).

Virus isolation: In total 587 cloacal swabs were grouped as 114 specimens. By inoculation in embryonated chicken embryos (ECE) 9 – 11day old, one H12 Avian Influenza virus was isolated from a moscovy duck flock.

Table 1. Prevalence of sera seropositive for H5 antibody in relation to different factors

Factors	Species/factors	H5 positive	H5 negative	Percentage	OR	p-value	95% CI
	Chickens						
	< 2 months	0	28	0	inf	0.5833	inf
	> 2 months	15	174	7.94	1.90	0.2476	[0.75, 4.77]
	Layer	7	154	4.35	1	-	-
	Ducks						
Bird ages	< 2 months	2	6	25	1	-	-
	> 2 months	1	3	25	1	0.4984	[0.06,15.99]
	Layer	56	8	87.5	21.00	0.0002	[3.60,122.49]
	M. ducks						
	< 2 months	9	3	75	10.83	0.0003	[2.71, 43.29]
	> 2 months	23	83	21.7	1	-	-
	Layer	0	15	0	inf	0.0997	Inf
	Chicken						
	Chicken flock	18	115	5.4	1.60	0.5790	[0.51, 5.02]
	Duck flock						
	Mixed flock	4	41	8.89	1	-	-
Mixed or	**Duck**						
	Chicken flock						
single	Duck flock	44	13	77.19	0.90	0.8745	[0.25,3.20]
flocks	Mixed flock	15	4	78.95	1	-	-
	M. duck						
	Chicken flock						
	Duck flock	20	77	20.62	0.52	0.1967	[0.22, 1.22]
	Mixed flock	12	24	33.33	1	-	-
	Chickens						
	Coastline	9	120	6.98	1.92	0.378191	[0.63, 5.89]
	Interior	8	128	5.88	1.60	0.598581	[0.51, 5.02]
	Riverside	5	128	4.42	1	-	-
	Ducks						
Geography	Coastline	14	1	93.33	5.79	0.160325	[0.68, 49.1]
	Interior	16	4	80.00	1.66	0.646461	[0.46, 5.99]
	Riverside	29	12	70.73	1	-	-
	M. ducks						
	Coastline	22	19	53.66	6.62	0.000305	[2.41, 18.2]
	Interior	3	42	6.67	0.41	0.353828	[0.10, 1.69]
	Riverside	7	40	14.89	1	-	-
	Chickens						
	Scavenging	1	34	2.86	0.11	0.029272	[0.01, 0.87]
	Backyard	5	262	1.87	0.07	0.000001	[0.03, 0.20]
	Indoor	16	60	21.05	1	-	-
Production	**Ducks**						
	Scavenging	27	4	87.10	2.25	0.469669	[0.52, 9.67]
system	Backyard	17	8	68.00	0.71	0.855736	[0.19, 2.64]
	Indoor	15	5	75.00	1	-	-
	M. ducks						
	Scavenging	3	2	60.00	4.17	0.324635	[0.60, 29.13]
	Backyard	20	74	21.28	0.75	0.704336	[0.30, 1.86]
	Indoor	9	25	26.47	1	-	-

3.3. Investigation on virus survivability/infectivity in different types of water

To estimate the survival/infectivity of a field isolation of AIV subtype H5N1 in aquatic environments, five sources of water were used including brackish water, arroyo water, pond water, buffed phosphate saline (BPS), and BPS with 1% milk. Virus was inoculated into water and incubated at 4°C, room temperature (20-25°C) and 37°C, respectly. The results are shown in Table 2.

Table 2. Effects of different water environments on virus inactivation under different temperatures in destroying infectivity.

Temperature	Inactivation status	Brackish water	Pond water	Arroyo water	BPS 1% milk	BPS
37°C (Incubator)	Inactivated	6 hr	6 hr	6 hr	ND	24 hr
	Not inactivated	<6 hr	< 6 hr	< 6 hr	> 4 days	18 hr
20-25°C (Room temperature)	Inactivated	6 hr	24 hr	90 hr	ND	66 hr
	Not inactivated	< 6 hr	18 hr	84 hr	> 4 days	60 hr
4°C (Refrigerator)	Inactivated	ND	ND	ND	ND	ND
	Not inactivated	>4 days	>4 days	>4 days	> 4 days	> 4 days

Note: ND = not done

When incubated at 37°C, it resulted in the loss of infectivity after 6 hours when mixed with pond water and arroyo water and brackish water, after 24 hours when mixed with BPS.

At room temperature, viruses survived longest in arroyo water (90 hours) and shortest in brackish water (6 hours).

When the virus was mixed with BPS 1% milk, it was not inactivated after 4 days following incubation at 37°C, 20-25°C, and 4°C.

4. Discussion

In this study, antibody against H5, H3 and H12 virus were detected in the serum samples; it means that these three subtypes of AIV must have been present in village poultry in Thai Binh province. In fact, H5N1 appeared in Vietnam during January 2004 as part of the big South East Asian outbreak of AI, H5N1 virus was initially detected in samples from a breeding

chicken farm in Thai Binh. No previous reports exist from Vietnam concerning H3 or H12 virus neither has AI virus isolation nor serology previously been attempted but H3 and H12 might be endemic in village poultry, appearing in an asymptomatic form. The low mortality and the few signs may cause confusion in relation to Newcastle Disease.

The finding of antibodies against AIV sub-types H5, H3, H12 in the same birds, at the same time indicates that these three virus subtypes have co-existed in Thai Binh village poultry. A significant difference between positive H5 antibody samples was found among different species of domestic poultry. In any flock type, geographical area and production system, ducks were confirmed being the species with the highest prevalence of H5 positive sera. The finding is in accordance with previous studies (Shortridge, 1997) indicating a high prevalence of AIV in domestic ducks as well as in wild ducks.

No H5N1 virus was isolated in the present study although sampling of 587 animals was performed. This may indicate that the virus was no longer found at the time of sampling. This would be in accordance with a recent study, which showed that H5N1 is shed for approximately 17 days (Anon, 2004_C). The present study was performed 20 days to one month after the last case was reported in Thai Binh province. However H5 has previously been shown to be difficult to cultivate (Jorgensen, personal communication)

The high prevalence of influenza antibody detected in ducks and moscovy ducks, the appearance of several AIV subtypes and the tradition of farmers rearing different poultry species in the same flock in all villages of Thai Binh province could pose a potential risk for the re-emergence of AIV in the future.

The disease appeared most virulent in chicken flocks, all infected chickens died, this might explain why the prevalence of H5 antisera was low in chickens in the present study. Moreover the finding of a high prevalence of H5 antibody in ducks in this study is in accordance with the results of a surveillance study by Shortridge. (1997). He found that the proportion of H5 virus isolations from ducks was higher than for any other AI virus.

During the outbreaks of AI in Thai Binh in 2004, ducks died in the beginning of the outbreak and thereafter mortality in ducks due to AI was seldom seen. The finding of a high seroprevelance of H5 antibody sera in ducks in scavenging systems and coastline areas in Thai Binh may be a potential risk factor for AIV transmission due to the to-and-fro among ducks flocks in scavenging systems, the density of duck flocks and the habit of farmers let-out their ducks scavenge on the field together.

However, in this study the chickens originating from mixed flocks were not seropositive to H5 to a higher extend than in single chicken flocks. This could be due to the fact that chickens die

following introduction of the virus by ducks. It is easy to understand that chickens will be in risk of getting infected when they have contact with ducks, which is a reservoir. A clear difference between the different types of ducks with regard to susceptibility apparently exists. But the mechanism explaining this is unknown.

The avian influenza virus can remain viable for long periods of time at moderate temperatures, and may survive indefinitely in frozen material. As a result, the disease can be spread through improper disposal of infected carcasses, manure, or poultry by-products. Lu et al., (2002) demonstrated that avian influenza virus could survive in chicken manure 8 - 12 hours at 28 - 30 °C and survive 20 days at 4 °C (refrigerator temp). Influenza virus may remain infective in lake water for up to 4 days at 22°C and over 30 days at 0°C (Webster at al., 1978). In experimental studies using different strains of avian influenza virus it was demonstrated that viruses might survive up to 207 days in water at 17 °C and up to 102 days at 28 °C (Stallknecht et al., 1990). According to Yi Guan, the 2004 H5N1 virus seems to have become more stable, it can survive in the environment for 6 days at 37°C, as compared to 2 days with the older strains (Normile, 2004).

In the present study it was demonstrated that H5N1 virus can survive for 6 to 90 hours at room temperature (15 - 25°C) but it will be inactivated within 6 hours following incubation at 37°C in surface water. The virus maintained its infectivity for at least 4 days at temperatures up to 25°C. This is in accordance with the results mentioned above and the consideration of Guan Y (Normile, 2004). Brackish water was collected from a coastline area so the salt-content in this type of water may be higher than in arroyo water. Resulted by Stallknecht et al., (1990) indicated that the duration of infectivity of influenza virus decrease when salinity and pH increase. The virus survived relatively long time in water having organic material (PBS 1% milk). Milk is considered to be rich in organic material and ensure a stable environment for the viruses. The survivability of the virus in arroyo water is probably also affected by this mechanism as this type of water is mixed with alluvium water which is fresh and contains organic material.

However, it should be stressed that the exact composition of the different waters types used in this study was not analyzed. Thus, it cannot be concluded which factors specifically were responsible for the demonstrated different survival time.

This investigation has its major importance as a descriptive study and further investigations should be carried out in order to determine the maximum survival time in relevant types of water. In addition, knowledge about the mechanisms by which e.g. the organic particles protect the virus is scarce and needs further investigation. The information that the virus is

capable of surviving more than 4 days in the water types where most of the ducks are kept in the rural areas of Vietnam and at temperatures encountered in Vietnam is very valuable. This information coupled with the fact that ducks may excrete H5N1 for up to 17 days and longer (Anon, 2004_c) illustrate the massive virus concentration, which may build up in the environment.

5. Conclusion

Many previous investigations from several parts of the world also have focused on seroprevalence and virus isolation of avian influenza virus in poultry, especially in chickens and ducks, but the present study seems to be the first concerning avian influenza in Vietnam and one of the few concerning rural poultry. Although the study was performed in a smaller geographical area and limited funds and laboratory equipments were available, several interesting observations concerning the serological response to AIV infections and the circulation of AI virus in rural poultry were made.

The observation that only few ducks died in the beginning of the Vietnamese outbreak coupled with the fact that a very high percentage of the ducks were positive for H5 antibodies clearly demonstrate the potential of domestic ducks to act as a reservoir for AI. Previous investigations have primarily focused on the role of wild web-footed birds in this sense. In addition, it was indicated that several AIV subtypes circulate in rural poultry in Vietnam. A significant difference in the serological response to AI between different types of commercial ducks was also observed.

Different water sources, relevant for poultry production in Vietnam, were shown to differ in their ability to influence survival and infectivity of the H5N1 virus. Such information may prove useful in future risk assessment analysis studies. The knowledge obtained in the present investigations substantiate previous epidemiological observations that heavy shedding by seropositive carrier birds to the environment in combination with a considerable ability of AIV to survive in the environment may be a major constraint to the control of outbreaks of AI

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Seroprevalence study and virus isolation of Avian Influenza in rural poultry of Thai Binh province – Vietnam

Seroprevalence study and virus isolation of Avian Influenza in rural poultry of Thai Binh province – Vietnam

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ABSTRACT: The seroprevalence of antibodies against Avian Influenza virus (AIV) and the possible circulation of AI viruses in domestic poultry in Vietnam were not investigated previous to an outbreak of (AI) H5N1 starting in December 2003. A cross sectional study was conducted, 587 blood and cloacal swab samples were collected from 7 villages of 5 districts presenting the three main geographical types in Thai Binh province (coastline, inland and riverside), chickens, ducks and muscovy ducks were sampled. Antibodies were detected by the HI test. The cloacal swab samples were inoculated to specific pathogenic free (SPF) embryonated eggs for virus isolation, HA tests was used for initial AIV detection and HI test for AIV sub-typing. In addition, reverse transcription-polymerase chain reaction (RT-PCR) was used for determining the HA and NA subtype of the virus.

Antibodies against H5, H3 and H12 were detected in the survey. A high proportion of the ducks was positive for H5 antibodies (77.6%), whereas only 5.8% of the chickens were positive, 24.1% of the muscovy duck tested were found positive. In contrast, H3 and H12 antibodies were common in chickens (36%, 30.7%). Only one influenza virus was isolated. It was from a muscovy duck and by RT-PCR and HI test; it was characterized as a H12 AIV.

Key words: Avian Influenza, seroprevalence, poultry, subtype, antibody, antisera, antigen, cloacal swab, muscovy duck,

1. INTRODUCTION

Recurrent epidemics of influenza are fairly predictable events and the highly pathogenic forms have been reported for the last ten years in many Asia countries. The Goose/GD/96-like virus was first isolated from geese in Guangdong province of China in 1996 (Cauthen at al., 2000). Latter on, the disease caused by H5N1 broke out in Hong Kong in 1997. The Goose/GD/96-like virus strain was later considered to be the origin of the H5 component of the 1997 Hong Kong H5N1 virus (Guan et al., 2002; Chen, 2004). From 1999 onwards, surveys of virus circulation from different avian species through blood testing, swabbing of cages and testing of fecal samples in Hong Kong showed that the number of H5N1 virus isolations from ducks and geese increased from 1999 to 2001 (Sims et al., 2003). A study of Tumpey et all. (2002) indicated that, an avian H5N1 influenza virus isolated from duck meat in May 2001 had been imported to the Republic of Korea from China. In 2002, HPAI was reported as causing mortalities of wild birds in Hong Kong (Anon. 2005). This was the first report of deaths in wild birds in Asia.

In January 2003, the mortality extended to waterfowl (flamingo) in Kowloon Park, Hong Kong and later that year (May 2003) the H5N1 virus was also detected in Japan in imported duck meat (www.ProMEDmail 12/05/2003). From the end of 2003 and up to now, H5N1 subsequently appeared in many Asia countries such as Vietnam, Thailand, Hong Kong, Malaysia, Laos, Indonesia and Cambodia.

High mortality was reported in many chicken farms in Tien Giang and Long An province in the South of Vietnam in December 2003. At the beginning, the local veterinary officials reported the disease as being caused by Pasteurella, a bacterium that possess a high virulence in South-East Asia region. Actions against the disease were applied but the disease re-occurred even in the Pasteurella vaccinated flocks. A suspicion of avian influenza as the cause of the disease was based on the Hemagglutinating characteristics of isolated virus and that no reactivity towards anti-Newcastle serum was observed. Using the serum specificity to AI virus in the Hemagglutination Inhibition test, the nature of the H5 influenza virus was then confirmed. Subsequently, the viruses were classified as H5N1 by RT-PCR (Dung, personal communication. 2004).

The disease appeared with typical clinical signs of HPAI including respiratory and CNS symptoms. High morbidity and mortality, especially in the layer flocks were observed. Chickens, ducks quails and muscovy ducks were the main poultry species affected and no wild bird cases were reported. Poultry kept in commercial systems were more frequently found

affected than backyard poultry. The infected farms were distributed in a patchy form and genetic viral analysis results suggested a by-bird-movement way of transmission and one infection source only. Moreover, the high virulence of the H5N1 virus was characterized not only by the sudden death of chickens with AI typical lesions but also by acute and severe infection in humans, 29 humans cases of AI were reported in Vietnam no cases were observed among of chicken farmers or veterinarians who were in direct contact with diseased birds. However, the human case appeared to be associated consumption of infected poultry (Dung, personal communication)

An observational study on AI in village poultry was carried out in Thai Binh province in the North of Vietnam. The study was aimed at obtaining information on seroprevalence to AI viruses in the province and on the AIV subtype diversity present in village poultry following the outbreak in 2003/2004.

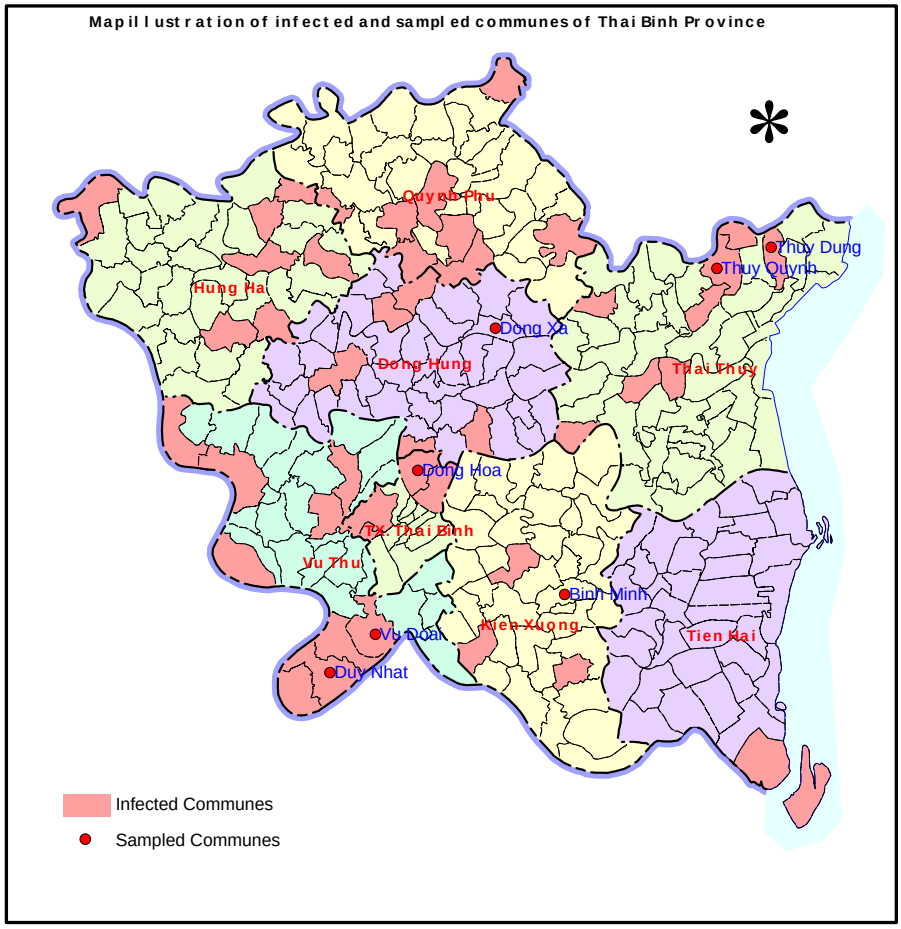
2. MATERIALS AND METHODS

2.1. Observation study

A cross sectional study was conducted during March 2004, in five districts of Thaibinh province, including Thai Thuy, Dong Hung, Thi Xa, Kien Xuong and Vu Thu. The districts included the three main geographical types of Thai Binh: coastline, interior and riverside. The climatic conditions are almost the same in all these districts but the water source is different. In the coastline area, the source of water is affected of seawater resulting in a high salt concentration. The riverside area is dominated by freshwater and usually influenced by floating of alluvial water. Due to the large land and water areas at these two locations, high population duck and muscovy duck rearing including laying ducks is common in these areas. The interior area is associated with ditch-water and poultry production consists mainly of chickens reared in backyards. Meat ducks are reared during the time of paddy harvest as the ducks can collect the fallen rice grains. Bird flu was reported in all types of districts during February 2004. The study was conducted approximately one month after the last reported outbreak in the province.

One hundred and six households, who reared chickens, ducks and moscovy ducks, were randomly selected from seven villages of the five districts. 4-7 birds were collected from each household for sampling. A blood sample was taken from each bird whereas cloacal swabs

from the same birds of household were pooled to make one specimen. In total, 379 chickens, 76 ducks and 132 muscovy ducks were sampled.



Five ml plastic syringes were used for bleeding; 1-1.5 ml of blood was taken from each bird, the samples were placed horizontally for the blood the blood to coagulate. Cloacal swabs were obtained using sterile cotton-tipped applicators (Hanoi medical material and equipments company) and placed in a sterile tub containing 2 ml transport medium supplemented with penicillin G (200 U/ml), streptomycin (200 µg/ml), polymycin B sulphate (100 U/ml), gentamycin (250 µg /ml), and nystatin (50 U/ml) (all from Sigma chemical company, st. Louis, MO).

Blood and cloacal samples were stored in a cool pack after sampling and shipped to Laboratory at Nation Institute of Veterinary Research (NIVR) on the same day or stored at 4ºC (blood) or – 20ºC (cloacal swabs) for shipping the following day.

The serologic test and virus isolation were performed at the laboratory for Avian Influenza at NIVR.

HI test. Sera were separated from all collected blood samples, treated at 56ºC for 30 minutes, and then tested for H5, H3, and H12 subtype antibody by the HI test. Selected samples were tested for antibodies against H9. No other antigens were commercial available at time of testing. The HI test was a standard beta test (Thayer & Beard, 1998), using 4 HA unit of antigen in 96-well plates, where the test serum had been two-fold diluted. The HI titer results were recorded as the reciprocal of the last serum dilution, which had complete inhibition of hemagglutinating activity.

Virus isolation. Each cloacal swab sample was inoculated into three embryonated specific pathogen free eggs (from Lien Ninh chicken breeding farm) in order to test for influenza virus (WHO, 1982) Influenza isolates were identified by hemagglutination (HA) test (WHO,1982). Negative samples following first passage were blind-passaged and it was considered negative when identification tests were negative at the second passage. HA-positive allantoic fluid (AF) was tested by hemagglutination-inhibition (HI) test (WHO,1982) and RT-PCR test (Speckman et al., 2002)

Data collection and analysis: The outputs were positive H5, H3 and H12 antibodies; and isolated viruses from blood and cloacal swab samples of village poultry in Thai Binh province. The factors included were bird ages, poultry species, flock types, production systems and geographic areas, which might influence the sero-prevalence and proportion of virus detection. The data was tested statistically by the SAS program using Chi-square test to compare two proportions in a 2 by 2 table in order to find the significant difference among related factors.

4. RESULTS

4.1. Seroprevalence of H5 antisera in domestic birds

In this study, 587 blood samples were obtained from 379 chickens, 132 muscovy ducks and 76 ducks (Table 1). Antibody to H5 was observed from chickens, ducks and moscovy ducks with titres ranging from 1*log*2 to 7*log*2. Antibody levels in chickens were significantly higher than in moscovy duck (53% of the samples had a H5 titre < 3*log*2) (Fig. 1).

As seen from figure 2, the prevalence of H5 antibody in ducks and moscovy ducks (77.6%, 24.1%) was significantly higher than in chickens (5.8%) ($p = 0.0001$). In addition, there was a

significant difference in prevalence between ducks (77.6%) versus moscovy ducks (24.1%) ($p = 0.0001$).

The prevalence of positive H5 antisera in relation to differences in age of the chickens, ducks and muscovy ducks is shown in table 2: The prevalence of H5 antibody in layer ducks (87.5%) was significantly higher than in young (< 2 month old) and adults (> 2 month old) ducks (25%, 25%). Although all of fifteen layers muscovy ducks were seronegative for H5, a high proportion of the young muscovy ducks (75%) were seropositive for H5 and the prevalence in adult was 21.7%. In contrast, the young chickens were seronegative.

The prevalence of positive H5 antisera in chickens, ducks and muscovy ducks, kept in the same household, were lightly higher than in households with chickens or ducks as only species. A significant difference between the two type of flocks is shown in the table 3; 8.89 % chickens, 33.33% muscovy ducks of mixed flocks had positive H5 antisera compared with 5.4%, 20.62% of chicken flocks and duck flocks only, respectively. The prevalence of positive H5 antisera of ducks in these two production systems was comparable.

The data shown in table 4 indicated that the disparity of prevalence of positive H5 antisera of chickens in coastline, interior and riverside areas (6.98%, 5.88%, 4.42%) was not statically significant. A similar situation was observed with ducks where 83.33%, 80%, 70.03% respectively of the ducks were seropositive. However, the prevalence of positive H5 antisera of muscovy ducks kept in coastline areas were significantly higher than in riverside and interior areas.

The proportion of chickens with positive H5 antisera kept in an indoor system was significantly higher than in scavenging and backyard systems. In contrast to the chickens, the proportion of muscovy ducks with positive H5 antisera in a scavenging system was significantly higher than in birds in indoor and backyard systems. Difference in seroprevalence was not observed between ducks of the three types of production systems (Table 5).

The prevalence of H5, H3 and H12 antibodies was also investigated in this study. The results are shown in table 6: The presence of these three antibodies could be demonstrated in the same flock or even the same bird. The prevalence of H5 positive sera was very high in ducks (77.63%); it was clearly lower in moscovyduck duck (24.06%) and lowest in chickens (5.82%). In contrast, the seroprevalence of H3 and H12 antisera in chickens (36.2%, 30.6%) was higher than in ducks (10.5%, 14.4%) and moscovy duck (17.2%, 5.2%).

4.2. Virus isolation

In total 587 cloacal swabs were grouped as 114 specimens. By inoculation into embryonated chicken embryos (ECE) 9 – 11day old, one H12 avian influenza virus was isolated from a moscovy duck flock.

5. DISCUSSION

Different subtypes of avian influenza cause disease in domestic poultry including chickens, ducks, quails, etc. Low pathogenic forms of avian influenza virus have circulated in domesticated and wild birds for many decades where little information on a possible reservoir for HPAI is available (ref), AI viruses include the following of HA subtypes H1, H2, H3, H4, H5, H6, H7, H9, H10, H11, H12 and H13 most of which have been isolated from poultry and wild birds (Alexander, 2003; Senne., 2003; Hanson et al., 2003). Outbreaks in Italy in 1997–1998 were caused by HPAI H5N2 (Capua, 1999). These outbreaks primarily occurred in chickens on small farms or backyard flocks, often with more species kept together. A HPAI virus subtype mutated from LPAI H1N7 virus in to HPAI in Italy in 1999 – 2000 and infected turkeys, chickens, guinea fowl, quails, ducks, and pheasants (Capua, 2003). A highly virulent H5N1 AIV was reported in Hong Kong in 1997 (Shorridge, 1999), subsequently, the virus re-emerged in 2001, 2002 and 2004. In addition, H5N1 was also reported as the agent of the big Asian outbreak in 2004 including Cambodia, China, Indonesia, Laos, Malaysia, and Thailand (Anon, 2004_a).

In addition, low pathogenic avian influenza virus H7N2 appeared in meat type chickens on Pennsylvania during 2001 – 2002 (Dunn et all., 2002). An H3N6 virus was isolated from caged birds in Singapore in 1997. Virus subtypes H4N6, H3N8, H6N2, H9N2, H11N9 and H13N6 were isolated in a surveillance study in ducks in Siberia and Hokkaido, Japan during 1997-1998, (Okazaki et al., 2000). Moreover, H3 viruses were reported from Great Britain (1997), Taiwan (2000), Denmark (2000), The Netherlands (2001), Portugal (2001) (Alexander. 2003). H3 viruses are also commonly isolated from pigs (Anon, 2004_b)

No H5N1 virus was isolated in the present study although sampling of 587 animals was performed. This may indicate that the virus no longer was excreted at the time of sampling. This could be in accordance with a recent study, which showed that H5N1 is shed for approximately 17 days (Anon., 2004_c). The present study was performed 20 days to one

month after the last case reported in Thai Binh province. However, H5 viruses have been reported to be difficult to cultivate (Jorgensen, personal communication. 2004)

In this study, antibodies against H5, H3 and H12 virus were detected in serum samples; it means that these three subtypes of AIV have been infecting in village poultry in Thai Binh province. In fact, H5N1 appeared in Vietnam during January 2004 as part of the big South East Asian outbreak of AI. Any report about the AI in Vietnam is available, the results of this study suggests that H3 and H12 subtypes might be endemic in village poultry, in an asymptomatic form causing low mortality or might have been miss-interpreted as Newcastle Disease or other respiratory pathogens. This study is one of the few, which clearly show that domestic ducks may be a significant reservoir.

The finding of antibodies against AIV sub-types H5, H3, H12 in the same birds, at the same time indicates that these three virus subtypes have co-existed in Thai Binh village poultry. A significant difference between positive H5 sera samples was found among species of domestic poultry. In any flock type, geographic area and production system, ducks were confirmed as being the species with the highest proportion of seropositive H5 birds. This finding is in accordance with previous studies (Shortridge, 1997) indicating a high prevalence of AIV in domestic ducks as well as in wild ducks.

A previous serological survey in birds from Southern Spain found that the percentage of ducks with influenza antibodies detected among wintering birds was high (Anrenas et al., 1990). This confirms that the central Mediterranean area play a key role in avian influenza ecology. A study by WHO in 2004 indicated that ducks might act as a silent reservoir for H5N1 virus. Ducks have probably played a central role in the march of H5N1 avian flu in Asia, where free-ranging chickens and duck flocks are kept together and share the same water (Becker, 2004). The high prevalence of influenza antibody detected in ducks and moscovy ducks, the appearance of several AIV subtypes and the tradition of farmers rearing different poultry species in the same flock in most villages of Thai Binh province could indicate an appropriate environment for AIV incursion and consequently the re-emergence of AIV in the future.

H5N1 influenza virus has been shown to be highly virulent; the viruses isolated from local farms in Hong Kong have shown to be highly pathogenic in standard pathogenicity tests (Anon, 2000), and it is capable of killing birds within 24 hours of inoculation (Normile, 2004). The mortality in affected flock develops quickly and goes up 100% (Sims et al., 2003). On the 6th of February 2004, 29 new outbreaks in Thai Binh province were recorded and 12,748 birds died or were culled (Source: Thai Binh Sub Department of Animal Health). The disease appeared mainly in chicken flocks, all infected chickens died, this might explain why the

prevalence of H5 antisera was low in the chicken investigated of the present study. Moreover the finding of a high prevalence of H5 antibody in ducks in this study is in accordance with the results of a surveillance study by Shortridge (1997). He found that the proportion of H5 virus isolation from ducks was higher than for other AI viruses. In the present investigation it could not be ruled out that other H5 viruses than H5N1 were involved as no N-typing was performed.

Many previous studies have indicated that higher isolation rate is observed in juvenile versus adult ducks (Hinshaw et al., 1980; Hinshaw et al., 1985; Alfonso et al, 1995 and Hanson et al., 2003). This study also finds a high seroprevalence of H5 antibody in layer ducks. However, the prevalence in muscovyducks less than 2 months was higher than in adult muscovyducks and in the chickens no difference related to age was observed. These results could be biased by a small sample size of the juvenile birds.

At present, many investigations indicate a significant role of ducks as a reservoir of AIV and source of AIV to other species. A finding in a laboratory experiment suggest that AIV can replicate well in the guts of domestic ducks without making them clinically sick (Source: WHO unpublished), and another survey from southern China show that asymptomatic ducks at live animal markets may shed H5N1 virus and suggests that ducks could be a key factor in the transmission of the virus. During the outbreaks of AI in Thai Binh in 2004, ducks died in the first month and thereafter mortality in ducks due to AI was seldom observed. The finding of a high sero-prevalence of H5 antibody in ducks in scavenging systems and coastline areas in Thai Binh may be a potential risk factor for AIV transmission and emergence of the disease due to the to-and-fro among duck flocks in scavenging systems, the density of duck flocks and the habit of farmers to let-out their ducks scavenging on the field together.

However, in this study the chickens from mixed flocks were not seropositive to H5 to a higher extend than in single chicken flocks. This could be due to the fact that chickens die following introduction of the virus to the flock. It is easy to understand that chickens will be in risk of getting infected when they have contact with ducks, which is considered a reservoir. The United Nation Food and Agriculture Organization also suggest that chickens and other poultry species should be raised separately (WHO, 2004). Keeping ducks and chickens apart would be impossible in Vietnam where free-ranging ducks and chickens mingle and frequently share the same water supplies.

Ducks and muscovy ducks are kept primarily on the coastline and in arroyo areas due to the potential natural feed resources on the field, ditch, and river. This may explain why the percentages of birds with influenza antibodies in these areas were higher than in inland area.

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ANNEX

Table:

Table 1: Prevalence of birds have antibody against H5 antigen (only considered with samples have HI titre from 3log2 and upper).

Birds	Survey birds	Birds (+)	(%)
Chicken	379	22	5,82 ± 1.2
Duck	76	59	77.63 ± 4.78
Mosc.duck	132	32	24.06 ± 3.71
Total	587	113	

Table 2. Prevalence of serepositive H5 in relation to age of chickens, ducks and muscovyducks

Species	H5 positive	H5 negative	Percentage	OR	p-value	95% CI
Chickens						
< 2 months	0	28	0	inf	0.5833	inf
> 2 months	15	174	7.94	1.90	0.2476	[0.75, 4.77]
Layer	7	154	4.35	1	-	-
Ducks						
< 2 months	2	6	25	1	-	-
> 2 months	1	3	25	1	0.4984	[0.06,15.99]
Layer	56	8	87.5	21.00	0.0002	[3.60,122.49]
Muscovy ducks						
< 2 months	9	3	75	10.83	0.0003	[2.71, 43.29]
> 2 months	23	83	21.7	1	-	-
Layer	0	15	0	inf	0.0997	inf

Table 3: Influence of structure of bird flocks to presence of H5 antisera

Flock types	H5 positive	H5 negative	Percentage	OR	p-value	95% CI
Chicken						
Chicken flock	18	115	5.4	1.60	0.5790	[0.51, 5.02]
Duck flock						
Mixed flock	4	41	8.89	1	-	-
Duck						
Chicken flock						
Duck flock	44	13	77.19	0.90	0.8745	[0.25,3.20]
Mixed flock	15	4	78.95	1	-	-
Muscovy duck						
Chicken flock						
Duck flock	20	77	20.62	0.52	0.1967	[0.22, 1.22]
Mixed flock	12	24	33.33	1	-	-

Table 4. Seropositive H5 sera obtained from difference geographic areas

Geographic area	H5 negative	H5 positive	Percentage	OR	p-value	95% CI
Chickens						
Coastline	9	120	6.98	1.92	0.378191	[0.63, 5.89]
Interior	8	128	5.88	1.60	0.598581	[0.51, 5.02]
Riverside	5	128	4.42	1	-	-
Ducks						
Coastline	14	1	93.33	5.79	0.160325	[0.68, 49.1]
Interior	16	4	80.00	1.66	0.646461	[0.46, 5.99]
Riverside	29	12	70.73	1	-	-
Muscovy ducks						
Coastline	22	19	53.66	6.62	0.000305	[2.41, 18.2]
Interior	3	42	6.67	0.41	0.353828	[0.10, 1.69]
Riverside	7	40	14.89	1	-	-

Table 5. Production systems contributing to prevalence of positive H5 antisera

Production systems	H5 negative	H5 positive	Percentage	OR	p-value	95% CI
Chickens						
Scavenging	1	34	2.86	0.11	0.029272	[0.01, 0.87]
Backyard	5	262	1.87	0.07	0.000001	[0.03, 0.20]
Indoor	16	60	21.05	1	-	-
Ducks						
Scavenging	27	4	87.10	2.25	0.469669	[0.52, 9.67]
Backyard	17	8	68.00	0.71	0.855736	[0.19, 2.64]
Indoor	15	5	75.00	1	-	-
Muscovy ducks						
Scavenging	3	2	60.00	4.17	0.324635	[0.60, 29.13]
Backyard	20	74	21.28	0.75	0.704336	[0.30, 1.86]
Indoor	9	25	26.47	1	-	-

Table 6. Seroprevalence of H5, H3, H12 antisera in village poultry

Virus subtypes	H5 positive	H5 negative	Percentage	OR	p-value	95% CI
Chickens						
H5	22	357	5.82	0.14	0.000001	[0.09, 0.23]
H3	136	243	36.24	1.27	0.143208	[0.94, 1.72]
H12	116	263	30.69	1	-	-
Ducks						
H5	59	17	77.63	20.5	0.000001	[8.89, 47.33]
H3	8	68	10.53	0.70	0.624916	[0.26, 1.84]
H12	11	65	14.47	1	-	-
Muscovy ducks						
H5	32	100	24.06	5.71	0.000033	[2.42, 13.49]
H3	23	109	17.29	3.77	0.003692	[1.56, 9.12]
H12	7	125	5.26	1	-	-

Table 7. Avian influenza isolation by inoculated to embryo egg (Passage1 & passage 2)

Inoculated embryo egg	Passage 1		Passage 2	
	Total	HA	Total	HA
Death egg before 24 hours	15	0	11	0
Death egg before 48 hours	61	1 (H12)	53	0
Death egg before 72 hours	32	0	25	1(H12)
Live egg after 72 hours	234	0	253	0
Total	342	1(H12)	342	1(H12)

Figure

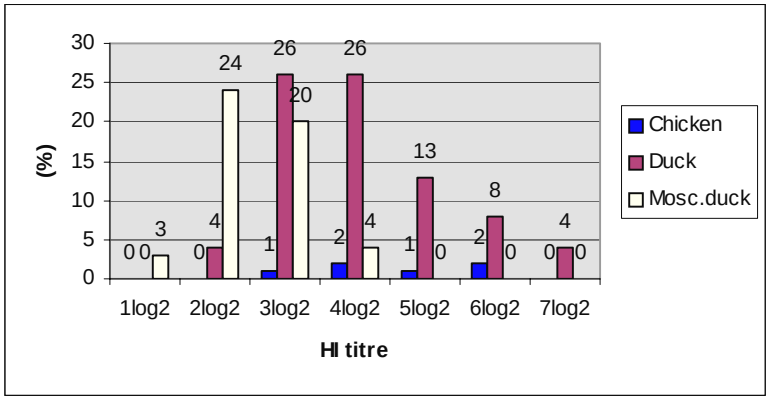

Fig. 1. H5 antibody levels in domestic birds

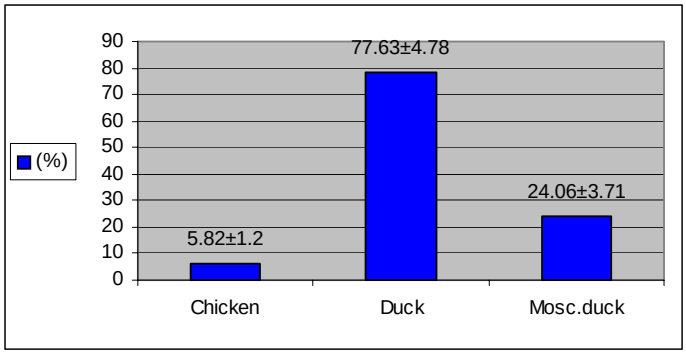

Fig. 2. Difference in H5 antibody distinguish among chickens, ducks and muscovy ducks

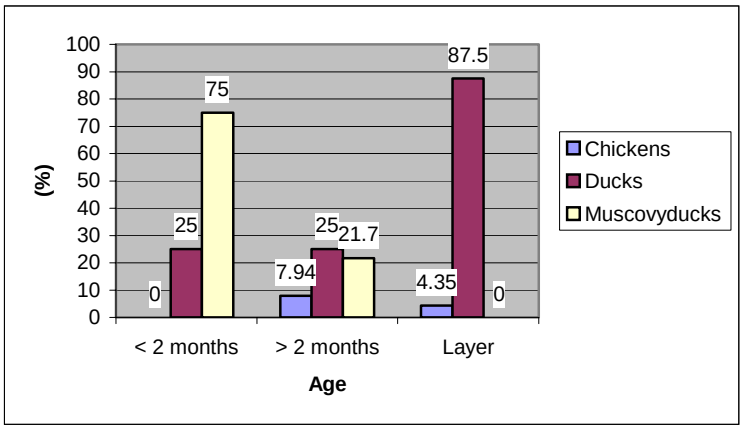

Figure 3. Prevalence of serepositive H5 in relation to age of chickens, ducks and muscovyducks

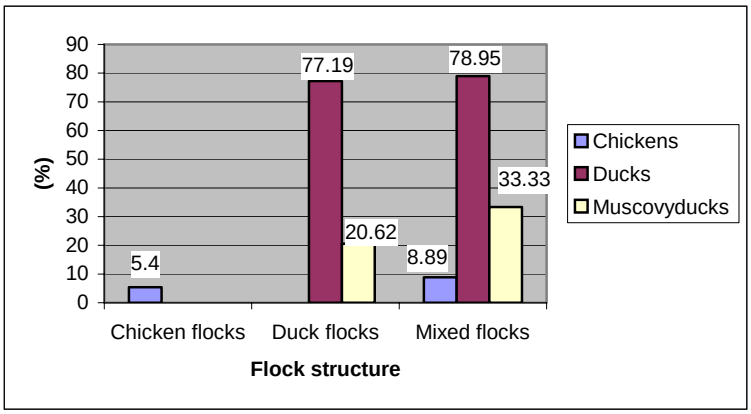

Figure 4: Prevalence of positive H5 antisera in difference flock types

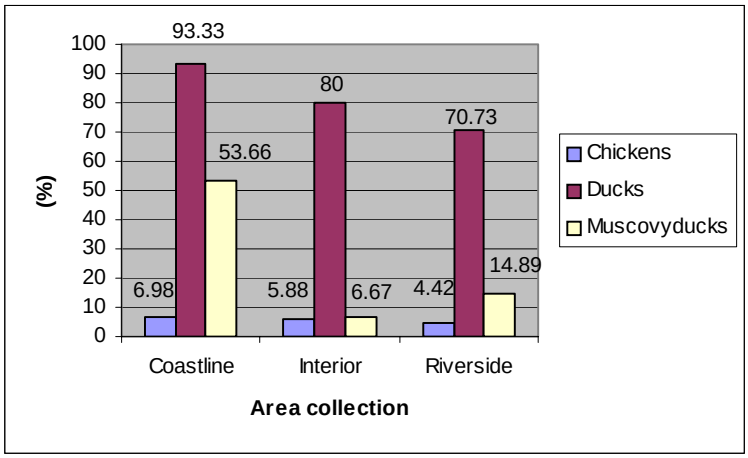

Figure 5. Seropositive H5 sera obtained from difference geographic areas

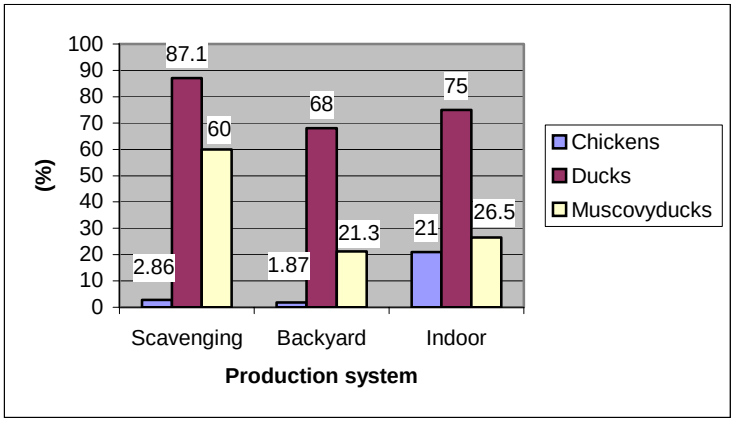

Figure 6. Production systems distribution to prevalence of positive H5 antisera

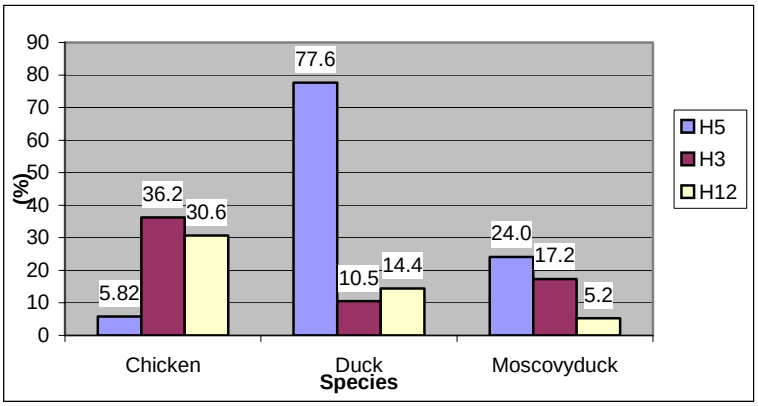

Figure 6. Seroprevalence of H5, H3, H12 antisera in village poultry

MANUSCRIPT

II

The survivability/infectivity of H5N1 avian influenza virus in different types of surface water

The survivability/infectivity of H5N1 avian influenza virus in different types of surface water

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ABTRACT: It is generally accepted that avian influenza virus (AIV) infected birds mainly excrete virus from the respiratory tract and intestinal tract to the environment. Therefore, factors such as water contamination with virus and persistence of virus in water and the fact that many bird species share water and food resources are considered to be of major importance in the transmission and spread of virus. An experimental study was performed investigating the effect of different types of surface water at difference temperature on the persistence of H5N1 avian influenza virus isolated from Tam Duong chicken farm of Vinh Phu province-Vietnam. The duration of infectivity of the AIV was studied by inoculation of the virus to brackish, pond and arroyo water collected from Thai Binh province. The infectivity of the virus was evaluated by observing cytopathogenic effects in Madin-Darby Canine Kidney (MDCK) cell culture. The H5N1 AIV was effectively inactivated at room temperature (20 – 25ºC) in brackish water after 6 hours, in pond water after 24 hours and in Arroyo water after 90 hours. AIV lost infectivity when incubated at 37°C for 6 hours in pond water, arroyo water and brackish water. However, the virus was not inactivated at day 4 at 4 °C in all three types of water. This suggests that environmental survival may play an important role in the epidemiology of avian influenza.

Key words: Avian Influenza, poultry, subtype, survival, cytopathogenic, shedding, environmental survival, effects.

1. INTRODUCTION

Highly pathogenic avian influenza (HPAI) H5N1 virus has caused serious threats for domestic poultry and humans in many Asian countries. The disease is characterized by high morbidity and mortality, respiratory and nervous signs, and it has spread quickly among flocks and been of major economic importance. The mechanisms by which influenza viruses are transmitted from one bird to another and bring about infection are poorly understood. Previous studies have shown that bird-to-bird transmission is extremely complex and depend on the strain of virus, concurrent infection species of birds, and environmental factors (Narayan et al., 1969; Alexander et al., 1978, 1986; Westbury et al., 1979, 1981).

According to Yi Guan. (2004), the virulence of the H5N1 virus is gradually decreasing in waterfowl because the virus cause rapid death and has less chance to reproduce (Normile, 2004). In both natural and experimental infections virulent viruses have tended to shown much poorer transmission from infected to susceptible chickens and turkeys than viruses of low pathogenicity (Anon, 2000). Hinshaw et al., (1980) found that the perpetuation of influenza viruses in Canadian free-living waterfowl was related to the passage of virus from adult to juvenile birds on lakes where the birds congregated before migration. A recent laboratory study of domestic ducks infected with several 2004 H5N1 viruses indicated that the quantities of virus excreted by the apparently healthy ducks approach those excreted by diseased chickens. This suggests that domestic ducks now might be acting as reservoir of the H5N1 virus, which is still highly pathogenic for chickens (Anon, 2004_a). Moreover, Webster et al., (1978) estimated the quantities of the virus excreted in feces to be up to $10^{8.7}$ mean egg infectious doses per g of feces from infected ducks. Infected birds excrete viruses to the environment in feces and respiratory exudations, this contaminates lakes, ponds or ditches, thus water may present a significant reservoir of virus. Hinshaw et al., (1979) have isolated AIV from untreated lake water where a large number of waterfowl congregated.

In addition, the survival time of AIV in natural water will play an important role in the transmition cycle of viruses. Webster et al. (1978) found that influenza viruses may remain infective in lake water for up to 4 days at 22°C and more than 30 days at 0°C. Stallknecht et al. (1990) estimated that from an initial concentration of 10^6 $TCID_{50}$ /ml the infectivity was maintained for up to 207 days at 17°C and 102 days at 28°C. However, few investigations have compared different types of water with regard to the ability to support virus survival. In Vietnam very different water sources can be observed in different parts of the country, thus it is very important to investigate their different properties concerning virus survival.

Thus, it is essential for disease control to understand how long infected birds excrete influenza viruses in feces and in oculo-nasal discharge and to determine the survival time of the virus in water. For that reason an experimental study was performed in order to elucidate aspect of the of H5N1 AIV survival in different natural water sources. The results may add to the understanding of the epidemiology of AI.

2. MASTERIALS AND METHODS

Preparation of complete Dulbecco's Modified Eagle Medium (D-MEM) with L-glutamine, TPCK-trypsin stock solution and Madin-Darby Canine Kidney (MDCK) cells in tissue culture flasks followed the procedures of the CDC manual.

Preparation of specimen: Five different diluents were used in this study: (1) Buffed Phosphate Saline (BPS), (2) 1% milk BPS, (3) bracket water collected from a coastline area, (4) ditch-water collected from interior area, (5) arroyo water collected from riverside area. A preparation of a water-virus mixture was added to a 50-ml sterile tube containing 18 ml water and 6 ml chorioallantoic fluid (CAFs) AIV (H5N1) (having an HA titre of 8 log2). The water-virus was mixed thoroughly and then the water-virus mixtures were transferred to a 5-ml sterile tube and incubated at 37°C, at room temperature (20-25°C), and at 4°C (refrigerator) respectively. The infectivity of the mixture of AIV was tested in MDCK cells every 6 hours by the following method:

An 0.5 ml diluent-virus mixture was transferred to an eppendorf-tube, centrifuged at 5000 X g for 10 minutes to separate sediment. A diluent-virus mixture was diluted in minimal essential medium (MEM) from 10^{-1} to 10^{-3} and then inoculated to MDCK cell at each concentration and was observed daily for cytopathogenic effect (CPE).

3. RESULTS

Five diluents were used to estimate survival/infectivity of a field isolates of AIV subtype H5N1 in aquatic environments. The main significant findings were the following (Table 1):

When the virus was mixed with brackish water, it lost infectivity following incubation at 37°C and 20°C - 25°C for 6 hours. At 4°C it survived for more than 4 days.

When the virus was mixed with ditch-water (pond water), it was inactivated after 6 hours following incubation at 37°C, after 24 hours at 20°C - 25°C, it survived at 4°C for more than 4 days.

When the virus was mixed with arroyo water, the virus lost infectivity when incubation at 37°C for 6 hours, at 20°C - 25°C for 90 days. The virus infectivity was still found after 4 days of incubation at 4°C.

When the virus was mixed with BPS, it was inactivated by the incubation at 37°C for 24 hours, at 20°C - 25°C for 66 hours, and it survived at 4°C more than 4 days.

When the virus was mixed with BPS 1% milk, it survived more than 4 days following incubation at 37 °C, 20°C - 25°C and 4°C

4. DISCUSSION

Influenza virus has been recovered from water and organic material from lakes and ponds where the infected ducks frequented (Jacob et al., 1998). Contaminated lake or drinking water may therefore be expected to result in infection by the faecal/oral route in susceptible birds. For all birds the ingestion of infective faeces appears to be most important mode of transmission and co-mingling of wild birds with range-reared domestic poultry is a key factor in some outbreaks (Anon, 2000).

The avian influenza virus can remain viable for long periods of time at moderate temperatures, and can survive indefinitely in frozen material. As a result, the disease can be spread through improper disposal of infected carcasses, manure, or poultry by-products. Lu et al., (2003) demonstrated that avian influenza virus could survive in chicken manure 8 - 12 hours at 28 - 30 °C and survive 20 days at 4 °C. Influenza virus may remain infective in lake water for up to 4 days at 22°C and over 30 days at 0°C (Webster at al., 1978). In experimental studies on different strains of avian influenza virus it was demonstrated that viruses might survive up to 207 days in water at 17 °C and up to 102 days at 28 °C (Stallknecht et al., 1990). According to Yi Guan, the 2004 H5N1 virus seems to have become more stable, it can survive in the environment for 6 days at a temperature of 37°C, as compared to 2 days with the older strains (Normile, 2004).

In the present study it was demonstrated that H5N1 virus can survive for 6 to 90 hours at room temperature (20 - 25°C) but it will be inactivated within 6 hours following incubation at 37°C in surface water. The virus maintained its infectivity for at least 4 days at temperature up to

25°C. This is in accordance with the results mentioned above and the consideration of Guan Y (Normile, 2004). In contrast the virus lost its infectivity relatively quick in brackish water. The brackish water was collected from a coastline area and consequently, the salt-content in this type of water may be higher than in arroyo water. Stallknecht et al., (1990) also indicated that duration of infectivity of influenza virus decrease when salinity and pH increase. The virus survived relatively long time in diluent having organic material (PBS 1% milk). Milk is considered rich in organic materials and ensures a stable environment for the viruses. The survivability of the virus in arroyo water is probably also affected by this mechanism as this type of water influences by alluvium water which is fresh and contain organic material.

However, it should be stressed that the exact composition of the different waters types used in this study was not analyzed. Thus, it cannot be concluded which factors were responsible for the different survival time. However, this investigation clearly demonstrates that different water sources differ in their ability to sustain virus survival.

The investigation has the importance as a descriptive study and further investigations should be carried out in order to determine the maximum survival time in relevant types of water. In addition, knowledge about the mechanisms by which e.g. the organic particles protect the virus is scarce and needs further investigation. The information that the virus is capable of surviving more than 4 days in water types where most of the ducks are kept in the rural areas of Vietnam and at the temperature commonly encountered is highly valuable. This information coupled with the fact that ducks may excrete H5N1 for up to 17 days and longer (Anon, 2004_b) illustrate the massive virus concentration, which may build up in the environment.

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ANNEX

Table 1. Effects of different water environments on virus inactivation under different temperatures in destroying infectivity.

Temperature	Inactivation status	Brackish water	Pond water	Arroyo water	BPS1% milk	BPS
37°C (Incubator)	Inactivated	6 hr	6 hr	6 hr	ND	24 hr
	Not inactivated	<6 hr	< 6 hr	< 6 hr	> 4 days	18 hr
20-25°C (Room temperature)	Inactivated	6 hr	24 hr	90 hr	ND	66 hr
	Not inactivated	< 6 hr	18 hr	84 hr	> 4 days	60 hr
4°C (Refrigerator)	Inactivated	ND	ND	ND	ND	ND
	Not inactivated	>4 days	>4 days	>4 days	> 4 days	> 4 days