

Surveillance of low pathogenic Avian Influenza in Dhaka District



A Dissertation Submitted in Partial Fulfillment of the Requirements for the
Degree of Master of Science in Biotechnology

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August, 2014**

***Dedicated to
My
Parents***

Declaration

I solemnly declare that the research work embodying the results reported in this thesis entitled “**Surveillance of low pathogenic avian influenza in Dhaka district**” submitted by me has been carried out under joint-supervision of Professor Naiyyum Choudhury and Dr. Md. Giasuddin in the National Reference Laboratory for Avian Influenza (NRL-AI), Bangladesh Livestock Research Institute, Savar, Dhaka. It is further certified that the research work presented here is original and has not been submitted to any other institution for any degree or diploma

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Abbreviation:

AI:	Avian Influenza
HPAI:	Highly pathogenic avian influenza
LPAI:	Low pathogenic avian influenza
rt-PCR:	Real time PCR
PCR:	Polymerase Chain Reaction.
HI:	hemagglutinin Inhibition.
FAO:	Food and Agricultural Organization.
NTP:	Nucleotide Tri Phosphate.
DNA:	De-oxy Ribonucleic Acid
RNA:	Ribonucleic Acid.
RLT:	Real life testing
RPE:	RNA Precipitating Elution
PPR:	peste des petits ruminants
TAE:	Tris-acetate-EDTA
OIE:	Office International des Epizooties
NP:	Nucleoprotein
vRNA:	Viral RNA
PBS:	Phosphate Buffer Solution
e.g. :	For example.
<i>et al.</i> :	And others.
rpm :	Revolution per minute.
M :	Matrix gene.

Abstract:

This present research was undertaken with a view to investigate the epidemiological and molecular study of Low pathogenic Avian Influenza (LPAI). For this purpose samples were collected from 800 clinically healthy birds of which 500 were migratory birds, 70 were ducks, 190 were commercial poultry and 40 from backyard poultry. For the migratory bird environmental samples (stool) were collected and for the rest of the birds cloacal swab were collected. Samples were inoculated into chicken embryo and checked for embryo death. Allantoic fluid from the dead embryo was collected and hemagglutination test was done. HA test positive samples were subjected to RRT PCR for matrix gene of avian influenza to confirm influenza A. The influenza A positive samples for matrix gene were sent to FAO & OIE central laboratory for the detection of H5, H7, H9 gene of AI.

Out of 500 migratory bird samples 20 were found HA positive in which 8 was found influenza A positive (7 was H9 and one was H5). Out of 190 live bird market samples 12 were found HA positive in which 4 were found influenza A positive all of which were H9 gene positive. Out of 70 native duck samples 4 was found HA positive but none was influenza A positive. Out of 40 backyard poultry samples 2 were HA positive and none was influenza A positive.

From the above study it was concluded that low pathogenic avian influenza is present in clinical healthy poultry and migratory bird. It also has shown one isolated case of highly pathogenic avian influenza. This indicates future threat of epidemic of highly pathogenic avian influenza in birds of Bangladesh which might be transmissible to human being. This risk of disease transmission is of significant public health concern.

CHAPTER 1

INTRODUCTION

Preamble

Avian flu (or avian influenza, and commonly known as bird flu) is a highly contagious and economically devastating disease occurring mostly in chickens, turkeys, and other gallinaceous birds. The viruses are distributed worldwide and cause serious economic losses. Moreover, the viruses have been isolated from a wide variety of animals, including humans, pigs, horses, tigers, cats, and other felids, ratites such as ostriches, emus, and rheas and sea mammals and therefore, are of sparked concern now due to their fatality and zoonoses (Alexander, 2000).

Type A Influenza virus is a negative sense single stranded RNA virus in Orthomyxoviridae family and has been classified into subtypes by the surface proteins hemagglutinin (HA) and neuraminidase (NA). At present, sixteen HA subtypes and nine NA subtypes have been recognized. Each virion consists of three major subviral components, namely (i) a viral envelope decorated with three transmembrane proteins hemagglutinin (HA), neuraminidase (NA) and M2, (ii) an intermediate layer of matrix protein (M1), and (iii) an innermost helical viral ribonucleocapsid [vRNP] core formed by nucleoprotein (NP) and negative strand viral RNA (vRNA) (Alexander, 2000).

According to Swayne (2008) HPAI was first observed to cause highly lethal disease in Italian poultry in 1878. WHO (2010) reported HPAI outbreaks in birds but by now HPAI has been reported in different animals in more than 60 countries. Cases of HPAI in humans have been identified in 15 countries. Giasuddin et al (2013) reported that Bangladesh first experienced HPAI in early 2007 and the NRL-AI at BLRI diagnosed and confirmed the presence of H5 subtype virus near the capital Dhaka. Now it has spread to at least 51 of the country's 64 districts. Till May 2013, 558 outbreaks have been recorded of which 498 are in commercial chickens and 57 in backyard chickens. More than 2.00 million chickens were culled by May 2012.

Avian flu in wild and domestic birds can exist in two different forms – one that has low capacity for causing disease (low pathogenic avian influenza or LPAI) and that causes disease very easily (highly pathogenic avian influenza or HPAI). Low pathogenic (LPAI) viruses mainly cause respiratory illnesses in poultry and generally low mortality. Highly pathogenic (HPAI) viruses cause systemic disease, often resulting in high mortality in turkeys and chickens (Alexander 2000; Chen *et al.* 2006). H5 or H7 AIV can be either LP or HP, all other known HA subtypes have only LP forms (Duanet al. 2007).

AI viruses can be transmitted directly or indirectly by contact with infectious aerosols and other virus-contaminated materials. Frequent pathways for the transmission are respiratory or airborne and gastrointestinal routes. Webster reported that wild birds had played an essential role for new introduction of AI virus into domestic poultry. Once AI viruses penetrate domestic poultry flocks, wild birds may no longer play a crucial role for spreading of infections within and between flocks. Infections are mainly spread by movement of poultry and poultry products. Philippa et al (2005) reported that AI viruses can maintain in live bird markets, water sources or anywhere poultry have been present if good biosecurity measures are not put into practice. In some circumstances, infected domestic poultry can transmit HPAI viruses to wild birds which are in close contact. This occurrence may introduce a potential threat of virus transmission to other domestic poultry populations. Murti et al (2004) stated that migratory water fowl – most notably wild ducks – constitute the natural reservoirs of the virus. Poultry flocks (Chicken, ducks, turkeys, geese) are susceptible because the virus can spread rapidly through contact between a sick bird and a healthy bird. Wild birds may carry H5N1 from one area to another through the process of migration (Webster et al, 2004).

Swayne (2000) stated the pathology associated with infection with HPAI H5N1 in animals appears to depend upon the host and the infecting virus strains. In chickens and other galliforme poultry, HPAI viruses replicate widely in endothelial cells throughout the body resulting in edema and cyanosis of the head, hemorrhages of the feet, leg shanks and visceral organs, and lesions of multiple organ failure resulting in necrosis of the endothelium of blood vessels in heart muscle, brain, adrenal gland and pancreas. On the other hand low pathogenic avian influenza causes respiratory and gastrointestinal symptoms.

In the last 10 years there has been a progressive increase in the number of outbreaks of avian flu in poultry compared with the previous 40 years. The spread of the disease has raised great concerns for animal and public health as Bridges *et al.* (2002) stated that human infection is rare; however, the reported case-fatality is high. Early reported case-fatality in 2003 was approximately 50%.

This present study was done as part of ongoing surveillance to detect the prevalence of LPAI in healthy birds in different regions of Dhaka district.

In the practical ground, the study was aimed to determine the prevalence of low pathogenic avian influenza in different bird's population. But sample population excludes diseased birds to detect the prevalence of low pathogenic avian influenza in healthy bird in a period when epidemic is absent. This will help to find the circulation of low pathogenic avian influenza in different population of healthy birds. As low pathogenic avian influenza virus can mutate to be highly pathogenic avian influenza, this study might predict the danger of having an epidemic in future.

OBJECTIVES:

The aim of the study was to detect the prevalence of low pathogenic avian influenza virus in different population of birds. The present study was conducted with the following specific objectives:

1. To find the prevalence in different population of birds.
2. To do the virus subtyping to know which subtype of virus is present in the circulation.

HYPOTHESIS:

Low pathogenic avian influenza virus circulates in migratory birds, backyard poultry, commercial poultry and native ducks even when no epidemic of avian influenza is present. This low pathogenic avian influenza will mutate into highly pathogenic avian influenza when appropriate condition will be present. This will ultimately cause the epidemic of highly pathogenic avian influenza in poultry.

SCOPE AND LIMITATION OF STUDY:

Surveillance study should be done in a larger sample size which was not possible due to resource and time constraints. Any sample if found negative in the first inoculation should be inoculated second time which was not also possible due to resource constraint. To get the full picture of prevalence of low pathogenic avian influenza in Bangladesh we should randomly select large samples from several districts and collect samples from randomly selected locations. In this study sample collection of migratory bird and commercial poultry was done in winter season and rest of the sample collection was done in the early rainy season. This is one of the limitations of the study.

CHAPTER 2

LITERATURE REVIEW

Since its emergence in late 2003, HPAI H5N1 has attracted substantial public attention because the H5N1 virus has shown to cause disease in both animals and humans (OIE 2009a, WHO 2010). Although there is a substantial body of literature relating to the epidemiology of influenza viruses in both humans and animals, it is important to review the disease situation, surveillance, and control strategies from time to time because of changing environmental and ecological conditions. Updated information can be used to direct future research into AI epidemiology and devise more effective programmes to control the disease. It is also important to understand the current AI surveillance system and control strategies applied, and how these influence the development and course of HPAI H5N1 in world. This chapter starts presents an overview of the etiology and epidemiology of avian influenza and factors that may lead to the introduction, spread and persistence of HPAI viruses. The following sections review the emergence of HPAI, control and prevention activities, and surveillance strategies. Spatio-temporal patterns of HPAI H5N1 outbreaks between 2003 and 2007 are described utilizing routine surveillance data. This literature review is not intended to be exhaustive, rather to provide a context and background for the work that follows. Comprehensive literature reviews related to AI and disease surveillance have been provided by Capua & Marangon (2004), FAO (2004b), and Swayne (2008).

2.1. Etiology

Capua & Marangon (2004); Swayne (2008) stated that avian influenza (AI) is caused by type A strains of influenza virus. All AI viruses are members of the Orthomyxoviridae family. Type A influenza viruses are classified into different subtypes according to the antigenicity of their surface proteins, haemagglutinin (HA) and neuraminidase (NA).

Webster & Hulse (2004); Alexander (2007) reported that there are 16 HA (H1 – H16) and 9 NA (N1 – N9) surface protein types that potentially form 144 HA _ NA combinations, but only 103

of these combinations have been described. Various combinations have been detected in avian species.

OIE (2006) reported that AI viruses in poultry are classified as being either highly pathogenic (HPAI) or low pathogenic (LPAI).

Alexander (2000) demonstrated that the HPAI viruses are defined as those that kill 75% or more of 4- to 8-week-old chickens within ten days of inoculation.

Alexander (2007) reported that only H5 and H7 subtypes viruses can cause HPAI, although not all viruses of these subtypes are virulent.

Elbers *et al.* (2004) reported that outbreaks of HPAI H7N7 caused up to 100% mortality in chickens and ducks within a few days in The Netherlands during 2003.

Cardona (2005) reported that the LPAI viruses (defined as those that kill less than 75% of 4- to 8-week-old chickens within ten days of inoculation) can include any of the 16 HA and 9 NA subtypes. LPAI viruses often go undetected and cause no clinical signs of infection. In some circumstances, low pathogenic strains can result in losses for poultry producers. For instance, during 2001 – 2002, a low pathogenic H6N2 strain caused disease and production losses in infected chickens and turkeys in California, USA.

Pasick *et al.* (2006); Senne (2006) reported that H5 and H7 LPAI viruses may mutate to be highly pathogenic and infect domestic poultry easily.

2.2. Host range

Webster *et al.* (2006) reported that all possible combinations of 16 HA and 9 NA can infect avian species but many infections do not show clinical signs, and some species are more resistant than others.

Smith *et al.* (2006); Xu *et al.* (1999) reported that since H5N1 HPAIV was first detected in 1996 at a goose farm in Guangdong Province in China, this infection then might have spread in poultry of many countries in Eurasia and Africa.

Kida(2008); Manzoor *et al.* (2008) reported that since HPAIVs have been detected in migratory ducks found dead in China, Mongolia and other Eurasian countries in spring in 2005- 2010, it is, therefore, a serious concern that these HPAIVs may perpetuate in the lakes where migratory water birds nest in summer in Siberia.

Okazaki *et al.* (2000); Ito *et al.* (1995) reported that influenza A viruses of different subtypes were isolated from water of the lakes where migratory water birds nest in summer, even in autumn when wild water birds had left for the south for migration, suggesting that influenza A viruses are preserved in frozen lake water each year while the wild water birds are absent.

Obenauer *et al.* (2006) reported that the geographical separation of host species has shaped the influenza gene pool into largely independently evolving Eurasian and American lineages.

Okazaki *et al.* (2000) stated that the influenza virus isolates from fecal samples of ducks in their nesting lakes in Siberia phylogenetically belong to Eurasian lineage and closely correlate to those from birds, pigs and horses in Asia. It was also noted that these isolates closely correlated to the H5N1 influenza viruses isolated from chickens and humans.

Kida (2008); Kida *et al.*(1979) stated that the phylogenetic analysis of the HA of H5 influenza virus isolates from ducks in Japan revealed a close relationship with those of H5N1 influenza viruses from Hong Kong, southern China, Thailand, and Vietnam indicating that the H5HA of these viruses originated from influenza viruses maintained in migratory ducks nesting in Siberia.

Guan *et al.* (2004) found that in addition to humans, domestic poultry and waterfowl, the infected host species for H5N1 has expanded to wild birds, canines, felines, swine and mustelidae.

Swayne (2008) stated that AI viruses circulating in wild birds through routine surveys and they generally are thought not to cause harm. Wild waterfowl are a natural reservoir of avian influenza A viruses, and these viruses are usually non-pathogenic in these species.

Makarova *et al.* (2003), Xu *et al.* (2007) reported that the quails may be an important reservoir because they are susceptible to different subtypes of AI viruses.

Hatta *et al.* (2001); Hoffman *et al.* (2001) revealed the effects of H5N1 not only adversely affected domestic bird and human population health, but also poultry industries on a global scale.

Kida *et al.* (1987); Kida *et al.* (1980); Tamura *et al.* (2007) stated that the influenza A viruses are widely distributed in birds and mammals including humans. Wild water birds are the natural reservoir of influenza viruses.

Oslenet *et al.* (2006) stated that among those, viruses of each of the known HA and NA subtypes (H1-H15 and N1-N9, respectively) other than H16 have been isolated from migratory ducks (the H16N3 virus has only been isolated once, from a seagull in 2005).

Kida (2008) stated that the ecological studies have revealed that a vast influenza virus gene pool for avian and mammalian influenza exists in migratory ducks and their nesting lake water and that influenza is a typical zoonosis.

Ito *et al.* (1985); Okazaki *et al.* (2000) stated that each of the known subtypes of influenza A viruses perpetuates among migratory ducks and their nesting lake water in nature.

Kida *et al.* (1980) reported that the influenza viruses have been isolated from freshly deposited fecal materials and from the lake water, indicating that migratory ducks have an efficient way to transmit viruses, *i.e.*, via fecal material in the water supply. Experimental infection studies have established that influenza viruses preferentially replicate in the columnar epithelial cells forming

crypts in the colon of ducks, causing no disease signs, and are excreted in high concentrations in the fecal materials.

Choi *et al.* (2005), Thiry *et al.* (2007), Lipatov *et al.* (2008) stated that cross-species transmission of AI viruses can potentially cause infection in mammals including humans, hamsters, mice, pigs, ferrets, stone martens, dogs, domestic cats, tigers, leopards, civets, and macaques.

Wright *et al.* (1992) reported that the turkey influenza virus isolates in the USA from 1980 to 1989 contained genes of swine origin and there was evidence that re-assortment of viruses from turkeys and swine had occurred.

Olsen *et al.* (2006) reported that over 105 species of wild birds belonging to 26 families, especially wild waterfowls (Anseriformes and Charadriiformes), have been infected with a range of LPAI viruses with various HA/NA combinations.

Webster (1978) stated that not only are aquatic birds natural reservoirs for avian influenza A viruses particularly LPAI, but their migratory routes match the geographical distribution of the viruses.

Boon *et al.* (2007) stated that the wild terrestrial birds may contribute in the interspecies transmission and spread of H5N1 viruses due to their ecology, habitat, and interspecies interactions.

Stallknecht and Brown (2007) studied the epidemiology of the disease in an attempt to learn more about the pathways of disease transmission and to help develop better control and prevention plans, since outbreaks of HPAI H5N1 occurred in many countries in 2004. They concluded that interactions between the host, agent, and environment are important aspects of the epidemiology of wild bird avian influenza.

Guan *et al.* (2002); Nguyen *et al.* (2001); Shortridge *et al.* (1998); Sims *et al.* (2003) reported that poultry-Galliformes, such as chickens, turkeys, peafowl, and quail are susceptible to AI. Surveillance in Hong Kong and Vietnam of LBMs continually has yielded isolates of HP H5N1; however, the samples are often from clinically healthy birds.

Isoda *et al.* (2006) reported that the HP H5N1-infected chickens experimentally, develop severe respiratory distress within 24 h, and die within 48 h. Laboratory inoculation in quail using HP H5N1 isolates caused death within 2–3 days.

Ellis *et al.* (2004); Hulse-Post *et al.* (2005) stated that in surveillance general wild waterfowl and shore birds are the reservoirs of LPAI, and can replicate and shed these AIVs without signs of disease. When infected with HPAI, and in the case of HP H5N1, results from show that there are differences in clinical signs of infection in wild birds from the same order and that signs of disease are genus specific. Some birds, like mallard ducks, can carry and shed HP H5N1 without clinical signs for long periods of time, whereas other migratory birds, such as geese, muted swans, and herons often die from infection.

OIE (2005) reported that in human case definitions are based on hospitalized patients, which include those with extremely high fever, influenza-like symptoms involving the lower respiratory tract, gastrointestinal symptoms and encephalitis with exposure to, or a recent history of handling, poultry.

Bridges *et al.* (2002) stated that human infection is rare; however, the reported case-fatality is high. Early reported case-fatality in 2003 was approximately 50%; however, more recently reported case-fatality in Indonesia was approximately 80%. These findings should be viewed with caution, since not all cases are confirmed while others may not be reported at all. A study of poultry workers in Hong Kong reported that approximately 10% of the workers were found with antibodies to H5N1, though none presented with any signs or symptoms of disease.

Kandun *et al.* (2006) reported that the government workers, who participated in the 1997 epidemic response, had a 3% seroprevalence. Surveillance in humans is often in response to a severe respiratory case discovered in a healthcare facility or household and therefore the true prevalence of the population with subclinical infection of HP H5N1 is unknown. Familial case-clusters from Indonesia analyzed in 2006 did not result in the identification of index cases within families but found that only blood-related family members were infected with H5N1, even though there was no apparent difference in exposure between blood-related and non-blood-

related family members. These findings may suggest a genetic predisposition to infection with H5N1.

Yingst *et al.* (2006) reported that dead cats were found nearby confirmed H5N1-infected premises housing domestic poultry, and H5N1 was isolated from the gut, stool, and trachea in the cats.

FAO (2006) reported that that human influenza subtypes can infect pigs; there is no evidence of adaptation of H5N1 in pigs prior to circulation in human populations. During the current epidemic, there has been an unofficial oral report of an isolated infection in pigs in China, and a single report of H5N1 in pigs in Indonesia.

Choi *et al.* (2005) reported that eight of 3175 (0.25%) pig sera samples from Vietnam and Thailand were positive for antibodies to H5N1. Experimental infection of pigs did not produce transmission between infected and susceptible pigs.

Butler (2006) stated that the canines were identified as another possible host for HP H5N1 from surveillance studies in Thailand.

American Veterinary Medical Association (2005); Crowford *et al.* (2005) reported that the outbreaks of influenza in canines have occurred before and the subtypes were found to be closely related to influenza circulating among equines.

2.3. Mode of transmission

Kelsey *et al.* (1996) reported that AI viruses can be transmitted directly or indirectly by contact with infectious aerosols and other virus-contaminated materials. Frequent pathways for the transmission are respiratory or airborne and gastrointestinal routes.

Hatta *et al.*(2001); Li *et al.*(2004); Sakoda *et al.*(2010) reported that since the H5N1 virus infections have become endemic in poultry farms in some countries and caused accidental transmission to humans, H5N1 viruses are recognized as one of the candidates for the next pandemic.

Amonsin *et al.* (2006) reported that a cause for concern for further risk of disease spread is the infection of felines with H5N1. In Thailand, tigers fed infected poultry carcasses became infected and died.

Thanawongnuwech *et al.* (2005); Rimmelzwaan *et al.* (2006) reported that Tiger-to-tiger transmission was evident and feline-to-feline transmission has been confirmed experimentally.

Suarez (2005) reported that in poultry the greatest amount of virus is shed 2 – 3 days after infection.

Lipatov *et al.* (2008) stated that in pigs, AI viruses are excreted only from the respiratory tract 1 – 5 days after inoculation.

Webster *et al.* (1978) stated that most AI viruses have previously been found to preferentially replicate in the gastrointestinal tract of birds and transmitted primarily via the oral-fecal route.

Sturm-Ramirez *et al.* (2005) reported that HPAI H5N1 viruses identified in the 2003 – 2004 epidemics in several countries in Asia have shown a reverse trend, with these viruses replicating at high levels in the trachea particularly in ducks.

Thus, the main path of transmission may have shifted from an oral-fecal route to more oral-oral route or even airborne route or both. This change may increase transmissibility of the virus. It may also influence the epidemiology of HPAI H5N1 and disease surveillance strategies.

Normile (2005) stated that some experts believe that HPAI H5N1 virus spread simply by movement of domestic poultry and contamination of fomites, however wild birds, especially migratory wild birds, may have carried the disease over long distances.

Kida *et al.* (1980) reported that the ducks are orally infected with influenza viruses by waterborne transmission at their nesting lakes in Siberia, Alaska and Canada around the Arctic Circle during their breeding season in summer.

Garamszegi and Moller, (2007); Webster, (1998) reported that avian influenza is transmitted via the fecal-oral route and this could be via direct contact with infected birds or indirectly via contamination of the environment including water and feed.

Horimoto and Kawaoka (2005) reported that in areas of high poultry density, HPAI viruses can also be transmitted through the nasal and oral routes.

Cinatl *et al.* (2007); Dwyer (2008) reported that the transmission of avian influenza virus to mammals, especially humans, can occur through direct exposure with infected poultry.

Peiris *et al.* (2007) reported that humans can be infected by HPAI H5N1 directly from sick poultry that excrete viruses in their faeces or through exposure to secretions through handling, slaughtering, preparing, and/or consuming uncooked contaminated products.

Bridges *et al.* (2002) revealed that the risk of infection in humans increased in occupations with intensive exposure to poultry such as butchers.

Keawcharoen *et al.* (2004); Songserm *et al.* (2006a); Songserm *et al.* (2006b); Weber *et al.* (2007); Songserm *et al.* (2006a); Weber *et al.* (2007) revealed that other mammals including tigers, a dog, and a cat have become infected after being fed infected poultry carcasses.

Guan *et al.* (2002); Liu *et al.* (2003) reported that the evaluation of isolates collected from surveillance in Hong Kong SAR and Mainland China LBMs concluded that LBMs provide an environment for AIV reassortment.

Mounts *et al.* (1999) conducted a case-control study in 1997 to determine risk for H5N1 infection found a statistically significant association with exposure to LBMs, but not with the preparation or consumption of poultry.

Ferguson *et al.* (2004); Yuen *et al.* (2005) reported that since 2003, poultry-to-human transmission of H5N1 has been found to be associated with direct contact with sick poultry, while there is limited evidence for human-to-human transmission among family members.

Dinh *et al.* (2006) conducted a matched case-control study in Vietnam in 2004. The authors reported that exposure or contact with sick or dead poultry within the house or neighborhood and no indoor water source within the household were statistically significantly associated with H5N1 infection.

Quan (2005) reported that though LBMs or live animal markets in general can pose a risk for AI infection, LBM exposure has not been associated with human cases during the current wave of the H5N1 epidemic. Another route for possible spread of H5N1 across national borders is illegal trade and transport of infected poultry or exotic birds. In some counties with H5N1 cases, where the demand for poultry is high, despite known risks of H5N1 transmission, poultry is transported illegally. Authorities in Vietnam estimate up to 70% of poultry that are illegally transported from China, go undetected.

Kilpatrick *et al.* (2006) stated that there are countries that have reported H5N1 infection in poultry in which infections are not associated with migratory bird movements and did not report

poultry trade with other reported infected countries. This has led some researchers to suspect illegal trade of poultry or poultry products as a source for H5N1 outbreaks.

Gilbert *et al.* (2006) reported that generally, wild waterfowl and shorebirds are the reservoir hosts of LPAI viruses. It remains a debatable issue among AI and wildlife experts of the capability and level of risk for long-distance transmission of a poultry-adapted virus, like HP H5N1, from infected wild birds.

Songserm *et al.* (2006) revealed that the poultry cases in Thailand were largely associated with rice patties and free grazing ducks in the area. The authors suggested that infection in domestic poultry was due to the co-mingling of wild waterfowl and domestic ducks arising from this type of agricultural practice.

Gilbert *et al.* (2006) reported that the poultry H5N1 outbreaks in Russia, Kazakhstan, and Turkey (Baltic Sea) all correlate in space and time with migratory bird movements.

Hulse-Post *et al.* (2005); Kishida *et al.* (2005) reported that dead wild bird infected with H5N1 in European countries have often been followed by isolated H5N1 poultry cases, further strengthening the suspicion that infected wild birds have spread H5N1. It is likely, through findings from experimental studies, that ducks are sub clinically shedding virus and likely spreading infection, not only to poultry, but also susceptible wild birds, such as swans, geese, and herons.

Gaidet *et al.* (2006) reported that the surveillance in Africa of wild birds has not yielded H5N1.

Hagemeijer *et al.* (2006) reported that migratory birds that have flyways through China and Southeast Asia also share their paths with Australia. So far, there have not been any reports of H5N1 infections in Australia. Migratory flyways are also approximations; all flyways for all migratory bird species are unknown and some researcher's think that assumptions of migratory bird spread is premature based on available data.

Dinh *et al.* (2004) reported that there is no evidence that cats can spread disease to other species, including humans. There have been no observations of human H5N1 infection from exposure to infected cats.

2.4. Distribution (space and time)

2.4.1. Prior to wave I: 1999–February 2003

Asia/SE Asia: Isolates of H5N1 from commercial geese and ducks during this period were from sub-clinically infected birds. In May 2001, a severe increase in mortality in chickens due to H5N1 was reported in Hong Kong. An immediate decision was made to cull over a million chickens within the same month, resulting in no further reports of poultry cases that year (Guan *et al.* 2004).

However, outbreaks have occurred in poultry in Hong Kong every year since 2001, usually in the winter months, coinciding with an increase in imported poultry to meet the demand due to Lunar New Year activities (Smith *et al.* 2004). HPAI outbreaks in wild birds are rare; however, cases of HP H5N1 in wild bird have been discovered through surveillance. In late 2002, high mortality rates were observed in free flying wild waterfowl in two parks in Hong Kong. Isolates of HP H5N1 from Kowloon Park were found to have the genetic marker consistent with prior adaptation in land-based poultry, whereas the H5N1 isolates from Penfold Park were missing this genetic marker on the NA gene (Ellis *et al.* 2004). Laboratory results from these isolates suggest many genotypes of H5N1 were circulating among wild birds in Hong Kong. Surveillance of AI in waterfowl during 1999–2002 in Mainland China yielded 21 H5N1 isolates from apparently healthy ducks in Southern China (Chen *et al.* 2004). Experimental inoculation trials using these isolates demonstrated viral shedding from trachea and cloaca in ducks and caused death in inoculated chickens within 8 days (Hulse-Post *et al.* 2005).

In 2001, HP H5N1 was isolated in South Korea from frozen duck meat imported from Mainland China; however, no subsequent cases of H5N1 were detected in South Korea (Lu X *et al.* 2003).

During 2001, surveillance in urban LBMs in Hanoi, Vietnam yielded several subtypes of AIV, including an H5N1 isolate from an apparently healthy goose. Genetic analysis showed the HA gene was closely highly related to those from poultry isolates in Hong Kong; however, the NA gene did not present with the common trait of adaptation found isolates from land-based poultry and humans (Nguyen *et al.* 2005). These findings were published after human and poultry cases were reported in Southeast Asia in the summer of 2003.

In February 2003, three family members visiting Mainland China in the Fujian province, were treated for severe respiratory distress due to H5N1 infection after returning to their home in Hong Kong SAR. No other human cases were reported in Hong Kong SAR or Mainland China at that time (OIE 2007)

2.4.2. Wave I: December 2003–March 2004

Early in the epidemic, it was weeks between the detection of disease and confirmation of H5N1 infection. For example, in December 2003 and January 2004, cases of H5N1 were reported in Vietnam, which was the first country to report H5N1 as a cause of mortality and respiratory distress in humans since 1997. However, severe respiratory distress that required hospitalization occurred in late October 2003 and these cases were later confirmed as H5N1 cases in January, 2004. Soon after case reports from Vietnam, Thailand also confirmed human and poultry cases of H5N1. Unlike previous reports of infected poultry from the 1997 outbreak in Hong Kong SAR, mortality in infected poultry in Vietnam and Thailand was high, nearly 100% in infected poultry flocks (FAO 2004).

From December 2003 to March 2004, an outbreak of H5N1 was reported in three chicken farms and in a number of pet chicken flocks in Japan (Mase *et al.* 2005). The farms were between 150 and 250 km away from each other, and dead crows found near infected farms were also found to be infected with the same genotype H5N1. The isolates from this outbreak were all genetically similar, but very different from the genotypes that had spread across Southern China, Southeast Asia, Russia and Europe. The isolates were similar to another genotype from Guangdong Province in Southern China (Mase *et al.* 2005). Genetic studies conducted on the isolates from Japan and

Korea show that the genotypes of H5N1 that caused outbreaks in both countries were very similar to each other (Lee *et al.* 2005).

In February 2004, Hong Kong SAR and Indonesia reported cases in poultry, but not humans. By the end of March, there were 34 human laboratory confirmed cases, 23 of which were fatal. Between March and June 2004, no human cases of H5N1 were reported. Vaccination for H5N1 in commercial domestic poultry was implemented as a control strategy in Mainland China, Hong Kong SAR, and Indonesia (FAO 2007).

Reported poultry cases were generally backyard poultry in rural areas, usually not kept indoors or within confined areas. Over 80% of poultry in Southeast Asia, and 60% in China are backyard, free-range flocks (FAO 2006; Otte 2006). Over 100 million birds were culled in affected countries; however, these efforts did not stop the next wave of HP H5N1 infections in these and others countries.

2.4.2. Wave II: June 2004–November 2004

In June and July 2004, China, Indonesia, Thailand, and Vietnam reported a recurrence of H5N1 in poultry. Japan and Korea were declared H5N1-free, and did not report any cases during waves II or III. Compared with wave I, wave II affected fewer countries and cases were reported from fewer municipalities from infected countries than during wave I (FAO 2007).

In August 2004, Malaysia reported poultry cases in nine villages. No human cases were reported. Poultry cases were reported from Malaysia through November 2004. Surveillance in areas surrounding the outbreak within 10 km led to finding H5N1 among poultry, which were promptly culled (FAO 2007).

In October 2004, another H5N1 outbreak in tigers at a Thai zoo was reported in which 147 died or were euthanized. Tigers that died from infection had respiratory distress and severe pneumonia (Thanawongnuwech *et al.* 2005; Tiensin *et al.* 2005). There was evidence of tiger-to-tiger as well as likely infection after the consumption of AI-infected chicken carcasses as the

modes of transmission. Two eagles, hidden in tubes and illegally imported into Brussels from Thailand, were found in October to be infected with H5N1. No clinical signs were observed in the birds, which were later euthanized. The man who smuggled the birds did not have symptoms and tested negative for H5N1 (ProMed mail 2007).

2.4.3. Wave III: December 2004–May 2006

Two months later, human cases were reported in Southeast Asia, marking the beginning of the third H5N1 epidemic wave. Since then, human cases have been reported every month in Asia, Eastern Europe, Africa and the Far East. In Asia, human cases in Indonesia were first reported in July 2005, and continued well into the winter of 2006 (WHO 2006). The case fatality worldwide has remained at approximately 60%; however, the human case-fatality rate in Indonesia is higher, at about 77% (WHO 2006). China reported the first human cases during this wave of the epidemic and poultry cases in 9 provinces, followed by culling 20 million and mass vaccination in all birds to control the epidemic (Li *et al.* 2004; Smith *et al.* 2006).

In October 2005, 276 dead songbirds that were being smuggled from Mainland China by cargo ship were intercepted in a Taiwan harbor and found to be infected with H5N1. This event, combined with the findings of infected eagles in Europe, raises concerns and awareness of this mode of disease spread (ProMed-mail 2005).

During this wave of the epidemic, the number of known susceptible species, including wild birds, grew. In July 2005, three captive civets, which had clinical signs similar to those in infected felines, died of H5N1 infection in Vietnam (FAO 2006). This was the first report of H5N1 in this species and was a cause for concern in Asia, as civets are sold in live animal markets or wet markets, in which exposure to wet markets was also a risk for human infection of sudden acute respiratory syndrome (SARS), a zoonotic severe respiratory disease that originated in China. The source of infection remains unknown since animal surveillance within a 10 km area did not find any animals infected with H5N1. No other reports of H5N1 infection in civets have been made since July 2005 (FAO 2006).

During April 2005, over 6000 wild migratory birds died at Qinghai Lake in Central China. The dead birds were mainly bar-headed geese, gulls, shelducks, and cormorants. H5N1 virus was the only pathogen isolated. Two months later, China reported an outbreak of H5N1 in domestic poultry in Xinjiang AR, located at the northwest border of China with Kazakhstan and Mongolia (FAO 2006). Prior surveillance at various sites of live migratory waterfowl, including bar-headed geese, did not result in the isolation of H5N1 in 493 samples taken from June 2004 to May 2005 (FAO 2007; Chen *et al.* 2006). In experimental studies of the H5N1 isolates from dead birds at Qinghai Lake in April, mortality in chickens and mice was greater than that from experiments using previous H5N1 isolates (Li *et al.* 2005). The authors concluded this was a new variant of H5N1, more virulent than earlier H5N1 isolates from ducks in China.

In October 2005, H5N1 poultry cases were reported in Turkey and Romania, and infected mute swans were reported in Croatia and Hungary (FAO 2006). The first outbreaks of H5N1 in Turkey were in commercial poultry farms in the western region of the country followed by human cases in the eastern region of the country in December. Human cases occurred in children under the age of 15 years who had extensive exposure and direct contact with sick or dead poultry. It is likely that human cases emerged because of the close human-poultry contact arising when poultry were brought indoors during cold weather (Euro surveillance editorial office 2006).

In November 2005, a single dead flamingo in Kuwait was found to be infected with H5N1; however, no other cases were reported in the country. All poultry and human cases throughout December occurred in Turkey, China and Southeast Asia. In February 2006, backyard flocks infected with H5N1 were reported in Iraq and Nigeria. Among reported domestic poultry cases in Iraq, domestic cats were also found to be infected with H5N1 (Yingst 2006). Two human cases in Iraq were reported, a 15-year-old girl and her 39-year-old uncle. Dead infected swans were also found in Iraq and Egypt in February.

In February, an infected wild duck was found and shortly after, turkeys on a commercial farm were also reported to be infected with H5N1 in France. In Germany, dead domestic cats, and a severely ill stone marten were found to be infected with H5N1 in March on Ruegen Island, where many wild birds were also found to be infected with H5N1 (Eurosurveillance 2006). By

April 2006, dead infected swans were reported from the UK and Germany reported an isolated outbreak of H5N1 on a large farm of turkeys, geese and chickens (Eurosurveillance 2006). Outbreaks of H5N1 in poultry were also reported from Denmark and Albania in June and March, respectively (FAO 2007).

2.4.4. Wave IV: June 2006–December 2006

From June to December, most human cases of H5N1 were reported from Indonesia (24 cases), followed by Egypt (4), Thailand (3), China (2), and Iraq (1). Outbreaks among poultry were more widespread, as reports continued from Northern Africa, China, and Southeast Asia (FAO 2006). Indonesia continued to report human and poultry cases. In November and December, South Korea reported a reemergence of H5N1 infection in poultry farms.

2.5. Evolution of the different subtypes of avian influenza virus

Padtarakoson (2006) showed that the influenza viruses have previously caused serious outbreaks of disease in both humans and animals. Evolution of the viruses is driven by both mutation of individual viral genes (antigenic drift) and reassortment of gene segments from different influenza viruses into a new virus (antigenic shift).

Potter (2001) demonstrate that the antigenic drift occurs when minor mutations occur due to proof-reading errors in the viral RNA replication process and result in insertion of different amino acids in viral proteins which can alter antigenicity. Antigenic shift involves major gene changes resulting from reassortment of the 8 viral genes from each of two influenza viruses during replication in the same cell and results in the emergence of a genetically different virus from the progenitor viruses. A high mutation rate is an important characteristic of RNA viruses resulting in the emergence of new strains, adaptation to a range of hosts, and development of different forms of pathology/clinical disease. Influenza viruses are believed to have caused pandemics since AD1590. However, the influenza virus was first isolated only in 1932.

Cox and Subbarao (2000); Johnson and Mueller (2002) reported that the emergence of H1N1 (Spanish Flu) in 1918 was one of the most widely reported pandemics which spread worldwide resulting in the death of up to 60 million people.

Kida (2008); Kida *et al.* (1987) reported that each of the past pandemic strains emerged through the genetic re-assortment between the viruses of avian and human origins in the cells lining upper respiratory tracts of pigs.

Duan *et al.* (2007); Liu *et al.* (2004); Manzoore *et al.* (2008) stated that the re-assortment between viruses of Eurasian and North American lineages has been found in wild water bird populations, indicating that these two geographically segregated lineages represent mixing populations of viruses.

FAO (2008) stated that the Z-genotype of H5N1 has emerged as the dominate strain that has spread in Southeast Asia, China, and Europe.

OIE (2005) reported in contrast with human influenza viruses, certain avian influenza viruses have been shown to exist as low pathogenic (LPAI) or high pathogenic (HPAI) biotypes based on their ability to cause severe disease in domestic galliforme birds. To date the avian influenza A viruses that have shown the HPAI biotype in domestic poultry are predominantly in the H5 and H7 subtype, although two H10 viruses have been reported.

Swayne and Suarez (2000) reported outbreaks of HPAI caused by H5 and H7 avian influenza viruses have been reported sporadically since 1959 but not all H5 and H7 viruses have the HPAI biotype.

Webster (2005) reported occasionally zoonotic spread of H5 and H7 HPAI viruses has resulted in human infections and deaths, but there have also been human infections with LPAI viruses such as H9N2 viruses.

Becker (1966) reported one influenza virus subtype H5 (H5N3) isolated from a disease outbreak in common terns (*Sterna hirundo*) in South Africa in 1961 caused a high level of mortality and was the first report of significant deaths of avian influenza in a wild bird species.

WHO (2008); Yee *et al.* (2009) reported that the Hong Kong H5N1 HPAI outbreak was preceded in 1996 by a disease outbreak in geese in Guangdong province, China caused by A/goose/Guangdong/1/96 (H5N1) virus (GsGd).

Shortridge (1999) stated in 1997 during the outbreaks of H5N1 HPAI in galliforme poultry in Hong Kong, the virus spread to humans resulting in 18 cases of which 6 died.

Ellis *et al.* (2004); Webster (2005) reported although avian influenza virus subtype H5 is commonly isolated and usually does not cause disease in waterfowl species, strains of H5N1 HPAI viruses isolated since late 2002 have caused severe disease and sudden death in wild waterfowl and other wild bird species.

Ellis *et al.* (2004); Sturm-Ramirez *et al.* (2004) stated the first evidence of H5N1 infection in wild birds was reported in Hong Kong in 2002 where the virus killed a variety of wild waterfowl.

Peiris *et al.* (2007) stated the H5N1 HPAI virus that evolved and resulted in the massive epizootic from 2003 to the present, not only resulted in fatalities in both wild and domesticated birds, but also caused disease with a high mortality rate in humans and other mammals.

Alexander (2007); Webster (1998) stated that all subtypes of avian influenza, including combinations of H1-H16 and N1-N9, have been isolated from avian species.

Webster *et al.* (1992) considered to be wild waterfowl are the natural reservoirs as many subtypes of influenza viruses can be isolated from these species without evidence of clinical disease.

Alexander (2000a) demonstrated that LPAI viruses can be isolated from up to 15% of ducks and geese and up to 2% of other species of wild birds. However, HPAI viruses are rarely isolated from wild birds and usually emerge by mutation from LPAI after being introduced to domesticated poultry

Webster (1998) revealed that in 1998 a phylogenetic study of nucleoproteins demonstrated that all mammalian influenza viruses were probably derived from an avian influenza reservoir.

Webster (1998) revealed that influenza viruses in some host-specific lineages had evolved from avian influenza viruses and viruses from humans and pigs also showed evidence of evolution from the same origin. Moreover, sub-lineages of avian influenza viruses tend to show limited variation in a geographical region and are considered to be in evolutionary stasis.

Schaffret *et al.* (1993); Webster *et al.* (2007b) reported the water bird avian influenza (AI) viruses have been separated into two superfamilies; American and Eurasian clades.

Webster *et al.* (2007) studied the frequency and extent of amino acid changes in individual viral proteins has shown that mammalian influenza viruses have a higher evolutionary rate than avian influenza viruses.

Horimoto and Kawaoka (2005) stated that the occurrence of genetic re-assortment in influenza A viruses is generally related to the frequency of mixed infections with these viruses in nature.

Webster (1998) considered pigs are well known as intermediate hosts serving as mixing vessels for re-assortment of influenza virus as they can be readily infected by both avian and human influenza A viruses.

Yuen and Wong (2005) stated that with the numbers of human H5N1 cases, humans should now also be considered as potential mixing vessels, particularly with the increased chance of co-infection with human seasonal influenza strains.

Wilschut and McElhaney (2005) considered that the 1997 HPAI H5N1 was a triple re-assortment involving viruses from multiple avian species including geese, chickens, ducks, and quail and this virus was transmitted directly from avian species to humans.

Black and Armstrong (2006) reported an Indonesian family which had seven members infected by HPAI H5N1 with six fatalities, and a Vietnamese nurse who was infected after nursing a patient infected with HPAI H5N1.

Guan *et al.* (2004) demonstrated that H5N1 was widespread in outbreak regions as seen in Hong Kong and that re-assortment occurred through interspecies transmission which may have involved aquatic and terrestrial wild birds, poultry and indirectly human activity. After introduction into new hosts recent H5N1 HPAI viruses have shown periods of rapid evolution with multiple changes in the amino acid sequences in multiple viral proteins, although the HA and some internal genes of human strains have been relatively conserved.

Hiromoto and Kawaoka (2005) noted that six internal genes (PB1, PB2, PA, NP, M proteins, and NS proteins) of human H5N1 viruses showed variability in amino acid substitutions, even though the viruses were isolated in the same year and from the same geographical location. Thus, these amino acid sequences that were specific to human variants may play a role in the disease transmission directly from poultry to humans (Zhou et al. 1999).

Cinatl *et al.* (2007) also are considered that mutations may also occur at any time which could result in a human to human transmissible strain developing.

Alexander (2000b) considered that emergence of a new highly pathogenic H5N1 HPAI strain that was capable of human to human transmission would have the potential to cause a very serious pandemic in the human population.

Philippa *et al.* (2005) stated that H5 and H7 viruses are not all highly pathogenic; they can mutate from low pathogenic to highly pathogenic forms and can infect a number of avian species.

Alexander (2000) reported that outbreaks of H5N2 influenza viruses occurred in Pennsylvania, USA. For the period of April to September 1983, the disease spread throughout the chicken population with low mortality since the virus was of low pathogenicity. However, clinical infections with high mortality were reported in October of the same year and the virus was confirmed as highly virulent. H5N2 outbreaks then reoccurred in some northeast states of the USA through live bird markets.

Capua and Marangon (2004) reported that the occurrence of LPAI viruses mutating to highly pathogenic forms was also recognized in Mexico in 1994 and Italy in 1999.

Sturm-Ramirez *et al.* (2005) reported that control efforts should not only target HPAI viruses but also LPAI viruses in domestic poultry. Different AI virus subtypes may exchange their gene segments to generate reassortments that may produce potentially pandemic strains.

Xu *et al.* (2007) demonstrate that reassortment between H9N2 and H5N1 subtype viruses has generated reassortments of both subtypes that have circulated in Southern China.

Guan *et al.* (1999) demonstrate that Quails may have played an important role in facilitating the reassortment of the H5N1 virus in the 1997 Hong Kong epidemic.

Lipatov *et al.* (2008) proposed that HPAI H5N1 viruses in poultry can reassort in pigs with human influenza viruses and adapt to efficient transmission in humans.

In conclusion, mixing of different species is thought to increase the chance of reassortment and interspecies transmission of AI viruses. It remains difficult to predict further virus evolution, so strategies are required to prevent the evolution of AI viruses and the emergence of pandemics. Proposed measures include the separation of species, increased biosecurity, better understanding of the virus, and improved vaccination strategies.

2.6. Persistence of the virus in the environment

Persistence of H5N1 viruses in the environment, especially in water bodies, has been investigated in a range of studies.

Brown *et al.* (2007) demonstrated that two Asian HPAI H5N1 viruses persisted in water for moderate periods of time. The viruses in their trial persisted in water with salinities of 0, 15, and 30 ppt (parts per thousand) at 17°C for up to 26, 30, and 19 days respectively and at 28°C for up to 5, 5, and 3 days respectively.

Fouchier *et al.* (2007); Olsen *et al.* (2006) reported that influenza viruses can remain infectious in lake water for up to 30 days at 0°C and 4 days at 22°C.

Stallknecht *et al.* (1990) has been demonstrated that some LPAI viruses can remain infective in water for up to 102 and 207 days at 28°C and 17°C, respectively.

Songserm *et al.* (2005) studied on the persistence of H5N1 in Thailand and revealed that the virus in chicken feces was killed within 30 minutes of being placed in sunlight at 32-35°C. However, the virus could survive in chicken feces for up to 4 days in the shade at a temperature of 25-32°C, as well as in paddy fields for up to 3 days.

2.7. Source of infection and contributing factors for virus transmission

It has been difficult to determine the precise origin of most HPAI outbreaks. Wild waterfowl and live-bird markets have been proposed as the most likely sources of infection in many HPAI outbreaks. However, precise information on sources of infection has not been recorded sufficiently in numerous others.

Nishiguchi *et al.* (2005); Wee *et al.* 2006); Balicer *et al.* (2007) reported conducted outbreak investigations respectively in Japan, the Republic of Korea, and Israel, the route of entry or source of virus was not conclusively proven.

Truszczyński and Samorek-Salamonowicz (2008) reported that wild birds are carriers of LPAI viruses that may not be pathogenic for poultry and humans. However, if these birds can also survive infection with HPAI viruses, they may spread the pathogenic virus over long distances during migration.

Capua and Marangon (2004) reported that climate change and consequent variations in wild bird migratory routes may also influence the incidence of AI introductions in domestic poultry.

Webster *et al.* (2006) reported that wild birds have been claimed to play an essential role for new introduction of AI virus into domestic poultry. Once AI viruses penetrate domestic poultry flocks, wild birds may no longer play a crucial role for spread of infections within and between flocks. Infections are mainly spread by movement of poultry and poultry products.

Philippa *et al.* (2005) reported that AI viruses can maintain in live bird markets, water sources or anywhere poultry have been present if good biosecurity measures are not put into practice. In some circumstances, infected domestic poultry can transmit HPAI viruses to wild birds which are in close contact. This occurrence may introduce a potential threat of virus transmission to other domestic poultry populations.

Gilbert *et al.* (2008) stated that free-ranging ducks increase the risk of introduction and spread of AI viruses in Southeast Asian countries where ducks scavenge in rice fields under rice-duck production systems. The geographical focus of the scavenging system is not only around the villages where duck owners are living, but also distant villages. Ducks are often raised in rice fields to scavenge for leftover rice grains, insects and snails. Ducks can also be in close contact with village poultry during field movements. Hence, scavenging ducks may play an important role in transmission of HPAI viruses.

Although the precise route of introduction and spread of HPAI viruses have not been clearly defined in many affected countries, wild birds are considered to play a key role in the spread of HPAI viruses in the wild and contribute to the introduction of viruses into domestic poultry. Human activities, especially those associated with poultry management and trade practices, are considered a more likely mode for transmission of the virus between domestic poultry populations. Trade in poultry and poultry products may facilitate the spread of disease across international boundaries. Detailed epidemiological studies are needed to provide reliable knowledge on the routes of introduction and spread of HPAI viruses to poultry populations.

2.8. Clinical Findings and Pathology AI disease

Swayne (2000) stated the pathology associated with infection with HPAI H5N1 in animals appears to depend upon the host and the infecting virus strains. In chickens and other galliforme poultry, HPAI viruses replicate widely in endothelial cells throughout the body resulting in edema and cyanosis of the head and comb, hemorrhages of the feet, leg shanks and visceral organs, and lesions of multiple organ failure resulting necrosis of the endothelium of blood vessels in heart muscle, brain, adrenal gland and pancreas.

Webster *et al.* (2007a) showed that AI viruses caused no clinical signs and limited pathology in domestic ducks but recently some H5N1 viruses have induced severe HPAI in domestic ducks.

Pantin-Jackwood *et al.* (2007); Stallknecht and Shane (1988) stated that infection with avian influenza viruses appears to be species and age susceptible.

Fouchier *et al.* (2007); Webster *et al.* (1978) revealed that in wild birds, LPAI viruses, which normally cause no disease, preferentially replicate in the intestine and are then shed in the feces of infected birds.

Beigel *et al.* (2005); Chotpitayasunondh *et al.* (2005); Tran *et al.* (2004) reported on the initial human cases, H5N1 infected patients showed high fever, cough, shortness of breath, diarrhea, and pneumonia.

Peiris *et al.* (2007); Subbarao *et al.* (1998); Yuen *et al.* (1998) stated some human patients develop an Acute Respiratory Distress Syndrome (ARDS) and renal failure.

Choi *et al.* (2005) showed pigs experimentally infected with HPAI H5N1 viruses from Thailand and Vietnam developed mild clinical signs, but there was no evidence of transmission to in-contact pigs.

Perkins *et al.* (2002) reported that neurological signs are observed in experimentally inoculated geese and emus. Ducks generally do not show clinical signs when infected with LPAI. An experiment inoculating ducks with H5N1 isolates yielded mixed results.

Hulse-Post *et al.* (2005) found that an inoculation experiment using H5N1 isolates from 2003 to 2004 in ducks, the ducks did not show clinical signs or mortality with the exception of one isolate of H5N1 from a human in Vietnam.

Songserm *et al.* (2006a); Songserm *et al.* (2006b) reported that in Thailand the domestic dogs and cats after the consumption of H5N1 infected chicken carcasses indicate that they undergo systemic infection and die shortly after infection. They display clinical signs of high fever, panting, and depression, and there is evidence of multiple organ inflammation and necrosis post mortem.

Keawcharoen *et al.* (2004) reported that infected tigers and leopards in a Thai zoo displayed respiratory and neurological signs prior to death.

Govorkova *et al.* (2005) reported ferrets challenged with H5N1 virus developed clinical signs including high fever, anorexia, diarrhoea and neurological signs followed by death associated with histopathological changes of the brain, lung, and liver including necrosis, degeneration, and/or inflammatory cell infiltrates.

Isoda *et al.* (2006) stated that miniature pigs inoculated with three genotypes of HP H5N1 did not present with clinical signs and 2 of the 3 H5N1 genotypes did not replicate. A recombinant H5N1 was the only genotype that caused seroconversion in the inoculated pigs.

2.9. Molecular epidemiology

Cauthen *et al.* (2000); Webster *et al.* (2005); Xu *et al.* (1999) reported that the emergence of the HPAI virus subtype H5N1 in Hong Kong in 1997 was caused by a triple reassortant virus with the HA gene being contributed by Gs/Gd/96 virus, internal genes coming from an A/Quail/Hong Kong/G1/97 (H9N2)-like virus (Guan *et al.* 1999; Guo *et al.* 2000; Webster *et al.* 2005) and the NA gene from A/Teal/Hong Kong/W312/97 (H6N1)-like virus (Hoffmann *et al.* 2000; Webster 2005).

Connor *et al.* (1994); Matrosovich *et al.* (1999) reported that human and chicken H5N1 viruses found in Hong Kong in 1997 contained an avian-like receptor binding to SA alpha 2,3 Gal-containing receptors only, which is a specific characteristic of the HAs of avian viruses.

Gaunet *et al.* (2002) studied a phylogenetic analysis revealed that H5N1 viruses isolated from terrestrial and aquatic birds in Hong Kong in 2000 had HA, NA and some internal genes (like Gs/Gd/96 virus) that were related to other viruses isolated from aquatic birds.

Chen *et al.* (2004) stated that the H5N1 viruses isolated from ducks in the southern part of mainland China during 1999-2002 were also closely related to Gs/Gd/96.

Kou *et al.* (2005) reported a new genotype of the H5N1 virus (A/Tree sparrow/Henan/1/04 to A/Tree sparrow/Henan/4/04) in tree sparrows (*Passer montanus*) in China in 2004. This virus

contained HA and NA genes from Gs/Gd/96-like viruses, nuclear protein genes from the 2001 genotype A H5N1 viruses, and other internal genes from an unknown influenza virus.

Guan *et al.* (2002); Li *et al.* (2004) reported respectively the H5N1 HPAI viruses isolated from live poultry markets in Hong Kong in 2001 were classified into five genotypes (A, B, C, D, and E) and from live poultry markets and farms in Hong Kong and mainland China in 2002 into eight genotypes (V, W, X1, X2, X3, Y, Z, and Z+).

Viseshakul *et al.* (2004) studied molecularly and revealed that an isolate, A/Chicken/Nakorn-Pathom/Thailand/CU-K2/04, from poultry showed a high degree of similarity to human isolates during the same epidemic in early 2004.

Buranathai *et al.* (2006) revealed that characterization of the Thai H5N1 viruses isolated from a variety of species, including wild birds, cats, and tigers, from 2004 to 2006 showed they were also genotype Z viruses.

Buranathai *et al.* (2006) revealed that the Thai isolates of HPAI H5N1 showed only minor changes in their HA, NA, M, NS, and PB2 genes and that there was no evidence of human to human transmission or oseltamivir resistance.

2.10. Worldwide situation

Swayne (2008) reported HPAI, subtype H7, was first observed to cause highly lethal disease in Italian poultry in 1878.

WHO (2010) reported HPAI outbreaks in birds have now been reported in more than 60 countries. Cases of HPAI in humans have been identified in 15 countries.

OIE (2009b) reported between 2003 and 2010, HPAI H5N1 outbreaks were reported in Asia, Africa, Europe, and the Middle East, affecting wild birds, domestic poultry, human and other mammals. In some countries, HPAI H5N1 outbreaks have been detected in wild birds but not in domestic poultry.

WHO (2010) reported sporadic human cases of various AI subtypes have been reported in recent years (H5N1, H7N2, H7N3, H7N7, and H9N2). Although there was no direct contact between human cases of HPAI H5N1 and affected chickens, the occurrence of HPAI H5N1 outbreaks in chickens and human cases in Hong Kong in 1997 has attracted international concern due to the possibility that this might provide sufficient conditions to initiate an influenza pandemic in humans. Between 2004 and 2005, H5N1 human infections were confirmed by laboratories in Vietnam, Thailand, Cambodia and Indonesia

2.11. HPAI surveillance programmes

Depending on epidemiological considerations and available resources, countries may choose to apply surveillance approaches with particular priorities or intensive surveillance programmes. For instance, HPAI free countries may focus on surveillance at international borders and on wildlife to detect incursions of HPAI. For HPAI affected countries, surveillance priorities may focus on the understanding of epidemiology, ecology and evolution of HPAI strains. Different countries have different regulations concerning disease surveillance strategies. As a result, surveillance quality and the capacity of veterinary services also vary greatly between countries and regions.

Gaidet et al. (2006) reported that the surveillance programmes are not only restricted to an individual country's border but also to regional or inter-continental networks, especially for transboundary infectious diseases like HPAI. In 2006, a large-scale surveillance study of wild birds was launched in several countries of Eastern Europe, the Middle East, and Africa to evaluate the perpetuation of HPAI H5N1 virus in wild birds.

OIE (2009b) reported that according to guidelines of the European Union (EU), the occurrence of HPAI in poultry must be reported to competent authorities of the member countries, and outbreaks of HPAI infection must be reported to the European Commission via the Animal Disease Notification System. Under threat of HPAI H5N1 subtypes from Southeast Asia, intensified wild bird investigations have been conducted focusing on high-risk species and examinations of dead birds. To ensure early detection of the virus and enhance collaboration, veterinary authorities, national reference laboratories, and the EU Reference Laboratory liaise closely. As a consequence, these surveillance strategies have contributed to efforts to prevent major HPAI outbreaks in domestic poultry in EU member countries.

Suarez *et al.* (2004); Max *et al.* (2006) conducted various surveillance strategies in the Americas. During the H7N3 epidemic in 2003, Chile conducted nationwide surveys to evaluate the potential spread of the virus in poultry populations. Sentinel birds were placed in previously infected farms to monitor silent spread of the virus. Results from these surveys demonstrated that the influenza infections of poultry in Chile in 2002 were the first isolation of AI viruses in poultry in South America. Chilean viruses were distinct from existing H7 AI viruses, and a novel mechanism-recombination between genes of the LPAI virus resulted in a virulent shift to cause clinical disease in chickens.

Senne (2006) stated that the intensive active and passive surveillance programmes in the USA detected evidence of 13 H (H1 – H13) and 9 N subtypes of AI viruses in live-bird markets and poultry farms during 2002 – 2005.

Pelzel *et al.* (2006) reported that the Texas Poultry Federation conducted a serologic surveillance programme in participating commercial flocks during 1995 – 2004. Samples were taken from all areas of the state. However, the index flock of the 2004 HPAI H5N2 outbreak in Texas was not a participant in the surveillance programme.

Rawdon *et al.* (2010); Stanislawek *et al.* (2002); Frazer *et al.* (2009); Langstaff *et al.* (2009) reported that in general, the sensitivity of HPAI surveillance programmes in individual countries is greatly influenced by the level of testing and reporting capacity. Biosecurity authorities in

New Zealand have conducted many AI surveillance programmes in wild birds and domestic poultry. Though the country has never recorded a case of HPAI in wild and domestic birds, LPAI H5 and H7 subtype viruses have been detected in healthy wild waterfowl.

Rawdon *et al.* (2010) conducted a serological survey to determine LPAI infection status of chicken and turkey farms. No evidence of H5 or H7 viruses was detected in the surveyed farms at the time of sampling. However, H5 subtype positive samples were identified in three free-range chicken farms, suggesting a historical local exposure of these chickens to virus from wild birds.

Gilbert *et al.* (2006) reported that in Asia, community-based and participatory surveillance programmes have been applied for HPAI detection. In 2004, Thailand launched an intensive active clinical surveillance programme (the so-called 'X-ray' strategy) to gather information of HPAI situation in the country. Thousands of trained inspectors participated in this survey.

Normile (2007); Desvaux *et al.* (2008) reported that as a consequence, the number of reported outbreaks increased remarkably soon after the announcement of the X-ray survey. This suggests that HPAI viruses were still circulating in the country, and the increase in number of HPAI outbreaks were likely influenced by the intensity of surveillance activities. In Indonesia, a participatory approach for HPAI surveillance has been applied to track where and when HPAI H5N1 outbreaks have occurred. Trained teams formally diagnose AI in the field by using rapid tests. Trained teams of veterinarians visit door to door asking villagers about outbreaks among their backyard chickens, and then persuade their cooperation in disease control efforts. Information obtained from villagers provides details of the epidemiological features of HPAI and contribute to control strategies. Although community-based and participatory surveillance approaches appear to be successful in Thailand and Indonesia, these surveillance programmes required great investment and efforts for implementation at national level.

In summary, HPAI surveillance capacity has been enhanced to allow early diagnosis of HPAI cases in many countries. Appropriate surveillance strategies provide better understanding of risk factors and the epidemiology of HPAI, poultry production and marketing systems, and the ability

to assess the effectiveness of vaccination programmes. To ensure successful control and eradication of HPAI, a country should consider applicable surveillance approaches according to its own situation and available resources. Results of national surveillance should be shared to provide neighboring countries knowledge of regional disease and infection status. Some of the data sharing may go on through international organizations like the WHO, FAO, and OIE. The three organizations have jointly developed the Global Early Warning and Response System (GLEWS), a linking network to improve global surveillance system for zoonotic disease.

In order to strengthen the link between the official veterinary services and the key informants of the animal health sector, participatory approaches appear to be able to provide some value.

2.12. Participatory approaches for AI surveillance

Jost *et al.* (2007) stated that the participatory epidemiology refers to the involvement of particular group of people taking part in epidemiological research and disease surveillance. Participatory methods overcome some of the limitations of conventional epidemiological methods in animal disease surveillance.

In participatory research local people participate more actively; they have the option of seeking technical assistance according to their own perception of need rather than according to external assessment by professionals. In summary, participatory approaches ensure that local people are actively involved in defining problems, proposing solutions and implementing applications.

2.13. HPAI control and prevention

Sims (2007) reported that the different approaches have been applied to control HPAI H5N1 and other circulated strains of avian influenza outbreaks including stamping out, vaccination, movement control, closure of live bird markets, and enhancement of biosecurity. Massive control strategies have remarkably reduced the incidence of disease and outbreaks have been limited to certain areas in some countries. A stamping out policy has proven to be effective in preventing and eliminating establishment of HPAI H5N1 and also other strains of virus in developed

countries where poultry are raised under intensive conditions and veterinary capacity is able to detect infection early. However, stamping out may not be the main tool used in countries where the disease is endemic. In these situations alternative strategies must be applied.

Senne (2006); OIE (2009b) demonstrated that vaccination against HPAI can protect poultry by providing resistance to infection, reducing the number of susceptible poultry and minimizing virus excretion. Vaccination was a key strategy for controlling the HPAI H5N2 outbreaks in Mexico in 1994 – 1995 and Central America in 2001 – 2002. Outbreaks have not been recorded in those countries during recent years.

Capua & Marangon (2004); FAO (2007b); and Sims (2007) demonstrated that China, Indonesia, and Vietnam have used vaccination as a key strategy for controlling recent HPAI H5N1 outbreaks. In combination with other measures, these countries have achieved substantial success in disease control.

Sims (2007) reported that recently, vaccination has been applied in other countries including Russia and Egypt.

FAO (2007b) reported that a combination of stamping out, vaccination and movement controls have proven to be successful in controlling the HPAI H5N1 outbreaks that occurred in Nigeria and Egypt during 2006.

DLD (2006) reported that the approaches for disease control and prevention can be modified according to the disease situation of individual countries. For instance, during the first phase of HPAI H5N1 epidemic during January 2004, Thailand applied a massive stamping out policy where poultry were slaughtered on infected premises and surrounding areas within a radius of 5 kilometers. This distance was reduced to 1 kilometer for later outbreaks. When the incidence of disease was sporadic, only poultry on infected premises were slaughtered.

Sims (2007) stated that in Hong Kong, culling of the entire poultry population was applied in the 1997 HPAI H5N1 epidemic, while a combination of measures were applied in the 2002 – 2003

epidemic including culling of infected flocks, vaccination and movement controls. Both control strategies eliminated the virus.

OIE (2009a) reported that the destruction of infected and at-risk poultry, in combination with movement controls and other measures, can lead to success in prevention of secondary spread of the disease. However, it is costly and requires a large amount of resources. In a country where the disease is endemic, it may not be feasible to proceed with massive culling. In this situation, only infected flocks should be slaughtered, and vaccination considered a suitable option. With application of massive control strategies involving a combination of stamping out, movement control, screening, and disinfection of infected premises France, Sweden, Germany and Denmark were able to eradicate the emergence of HPAI H5N1 during 2006. However, the disease was re-introduced in Germany during 2007 – 2008.

FAO (2007b) reported that although the use of combined control measures including stamping out, enhanced biosecurity, and movement control had been applied, it was difficult to eliminate HPAI viruses in Russia, Kazakhstan, Albania, Azerbaijan, Serbia, and Turkey.

In summary, effective control and prevention strategies for HPAI require a combination of disease control methods. Early detection of incursions of virus can prevent establishment of disease in countries with proper surveillance systems. When the disease is emerging, massive culling of infected and at risk flocks is the appropriate response in countries with strong veterinary services and financial resources. In countries where detection of disease is delayed, disease may be widely spread at the time of detection. Control measures should take this issue into account. Most of the countries experiencing HPAI outbreaks have been able to eliminate the disease in poultry and prevent human infection. However, the re-introduction of disease has occurred in some countries recently. The disease may be difficult to detect and control in countries with deficient veterinary services and resources.

2.14. In Bangladesh-Situation

Giasuddin *et al.* (2013) reported that Bangladesh first experienced HPAI in early 2007 and the NRL-AI at BLRI diagnosed and confirmed the presence of H5 sub-type virus near the capital

Dhaka. Now it has spread to at least 51 of the country's 64 districts. Till May 2013, 558 outbreaks have been recorded of which 498 in commercial chickens and 57 in backyard chickens.

Giasuddin *et al.* (2013) also reported that More than 2.00 million chickens were culled by May 2012. The phylogenetic analysis of Bangladeshi isolates (from 2007 to 2010) Bangladeshi isolates clustered with those from middle China, South mid Asia, Middle -East, Europe and Africa popularly known as Qinghai Lineage or Euro-Asia Africa lineage. Only one clade 2.2 virus was circulating in Bangladesh. From 2011 it was observed that-introduction of two new clades 2.3.2.1 and 2.3.4 viruses in to Bangladesh in addition to clade 2.2 viruses since 2007. But It was observed (2012) that clade 2.2 virus is being replaced by clades 2.3.2.1 virus. Avian influenza is now endemic in Bangladesh.

CHAPTER 3

MATERIAL AND METHODS

3.1. Experimental Design

Surveillance samples such as fresh voided fecal materials, cloacal swabs were collected randomly from selected areas in Dhaka district during January 2013 to March 2014. A total of 800 samples were collected with virological transport media (PBS + antibiotic). Samples were transported and processed in the National Reference Laboratory for Avian Influenza (NRL-AI), Bangladesh Livestock Research Institute, Savar, Dhaka. After processing the supernatant fluids were collected and inoculated into 10 days embryonated chicken eggs for culture of the virus. Finally RNA was extracted using one step RNeasy kit of QIAGEN (Germany) according to manufacturer's instruction for the detection of avian influenza virus by rRT-PCR using appropriate primers and probe against matrix gene and detection of low pathogenic avian influenza (H9) by RT-PCR using H9 specific primers.

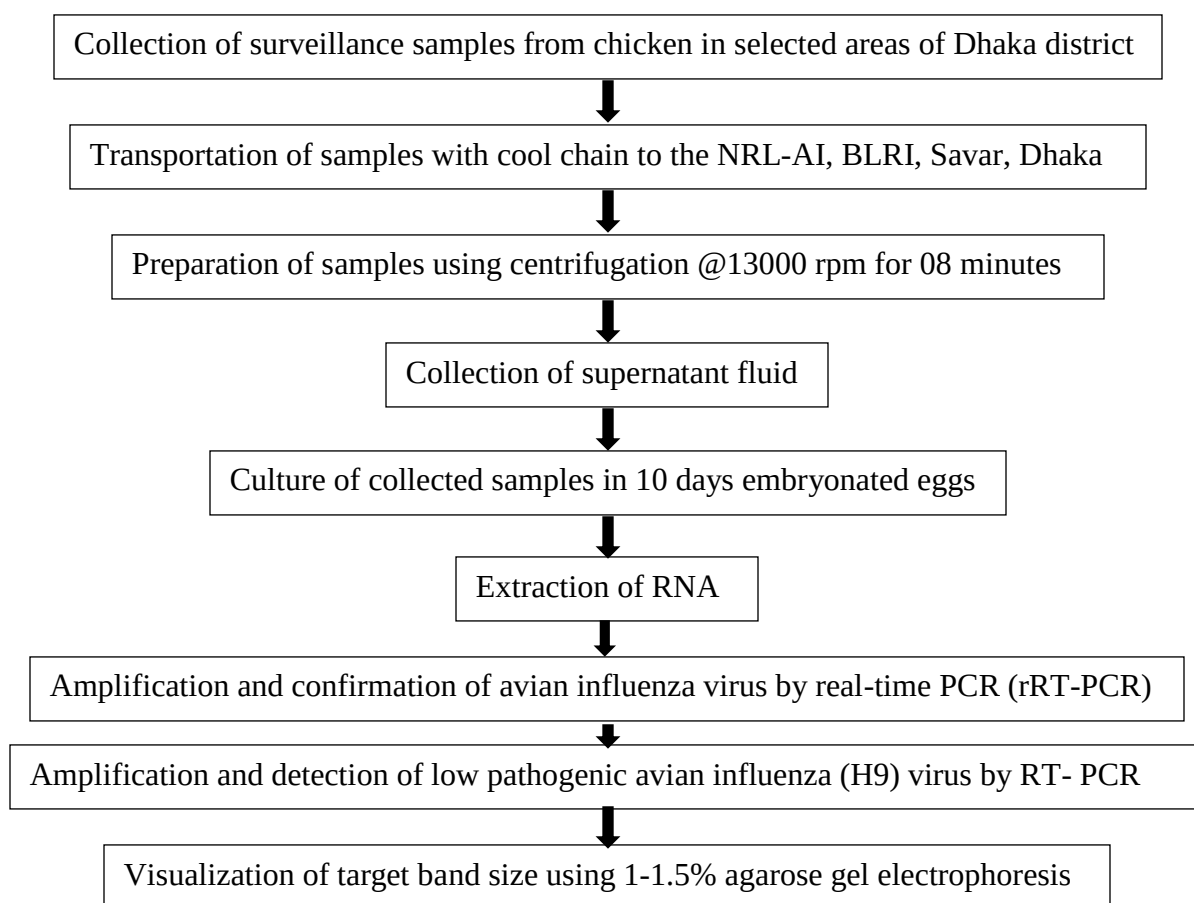


Fig 1: Schematic representation of experimental design.

3.2. Materials

3.2.1. Samples

Samples were collected in three different settings like household or backyard poultry, market bird and migratory bird. In the case of household poultry and market bird cloacal swabs were collected and for migratory bird freshly voided fecal samples were collected.

3.2.2. Materials used for sample collection

- Marker pen
- Ice box with icepacks
- Cotton buds
- Cryovials / polypacks
- Sample transport medium
- Pen
- Hand gloves

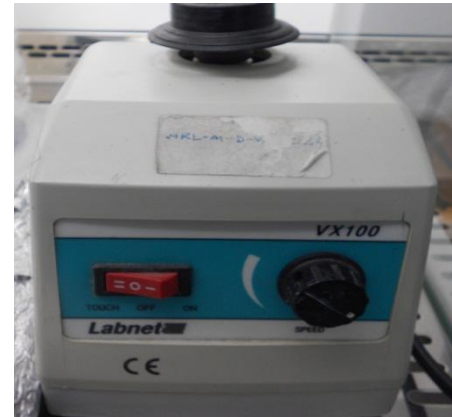


Fig 2: Vortexer

3.2.3. Materials used for sample preparation

- Class II Biosafety Cabinet
- Vortex machine (Thermoymne, Type 16700)
- Forceps
- Centrifuge machine (Eppendorf 1545 NWD)
- Syringe filter 0.22 μm (To exclude bacteria and other im
- Micropipette
- Eppendorf tube (1.5 ml)
- Tips
- Antibiotics (Gibco; Anti-Anti $\text{\textcircled{R}}$ 100X)
- Marker pen



Fig 3: Centrifuge machine

3.2.4. Materials used for the molecular detection of AI virus (RT-PCR Assay)

3.2.5. Viral RNA extraction kit

QIAGEN (Germany) RNeasy One-step Mini Kit (Cat No. 74106) was used for extraction of RNA from sample.

3.2.6. Materials used for RNA extraction

- Safety cabinet (Telster, AH-100 & Esco Class II)
- Falcon tubes (25 ml)
- RNase and Nuclease free Eppendorf tubes (1.5 ml)
- Micropipette (10, 20, 100, 200 and 1000 μ l) (pipetteman, Gilson S.A.S. France and eppendorf, Aurora, Finnpiquette)
- Sample
- PBS (Phosphate buffered saline)
- Alcohol (Pure and 70 %)
- Centrifuge machine (Eppendorf 1545 D)
- Vortex machine (Thermomixer, Type-16700)
- Micropipette tips

3.2.7. Reagents and kit used for viral RNA extraction

Except pure and 70 % alcohol other materials were obtained from QIAGEN (Germany) in the form of kit (RNeasy one step Mini Kit; Cat No. 74106).



Fig 4: One step RNeasy Kit
QIAGEN (Germany)



Fig 5: Reagents used for viral
RNA extraction

3.2.8. Reagents and chemicals used for preparation of reaction mixture (Master Mix)

- Nuclease free water
- 5X QIAGEN One Step RT-PCR buffer
- dNTP Mix (10 mM of each dNTP)
- Primer Forward (F)
- Primer Reverse (R)
- Enzyme Mix
- Template RNA

3.2.9. Primers

Two sets of primers were used targeting specific F and N genes of PPR virus. The nucleotide sequences of the primers and their references are given in Table 1. The lyophilized primer was reconstituted in nuclease free water to a concentration as per instruction of the manufacturer.

Table 1: Primers and probe Sequences used for Real-Time PCR

Matrix gene primers and Probes

Primer sequence	Source
Probe: FAM M+64 5'-FAM. TCAGGCCCCCTCAAAGCCGA-3'-TAMRA	E. Spackman <i>et al.</i> (2003)
Primer Forward: M+25 5'-AGATGAGTCTTCTAACCGAGGTCG-3'	
Primer Reverse: M-124 5'-TGCAAAAACATCTTCAAGTCTCTG-3'	

H9 gene primers and Probes

Primer Sequences (5' -3')	Amplicon size (bp)	Source
H9 Forward-151f 5'-CTYCACACAGARCACAATGG-3'	488	Lee <i>et al.</i> (2001)
H9 Reverse-638r 5'-GTCACACTTGTTGTTGTRTC-3'		

3.2.10. Materials used for electrophoresis

3.2.11. Equipments used for electrophoresis

- Gel electrophoresis apparatus (Gel X₂ Ultra V₂ Life Technologies)
- Micropipette
- Tips
- Micro oven (Alaska® Automatik)
- UV-Transilluminator (UVP-062807-001)

3.2.12. Reagents and chemicals used for electrophoresis

- Agarose (SIGMA, Batch No. 036K1643)
- Loading dye (QIAGEN® Gel Pilot loading dye)
- Ladder (QIAGEN® Gel Pilot 100 bp DNA ladder/100 lanes)
- Ethidium bromide (1 mg/ml)
- TAE (Tris Acetate EDTA) buffer.

3.3. METHODS

3.3.1. Collection of sample

A total of 800 samples were collected in three different settings like household or backyard poultry, market bird, migratory bird. In the case of household poultry and market bird cloacal swabs were collected and for migratory bird fresh voided fecal samples were collected.

3.3.2. Preparation of sample

Immediately after collection, the sample will be transferred to the Bangladesh Livestock Research Institute (BLRI) laboratory maintaining proper cool chain. Then the sample will be centrifuged at 3000 rpm and supernatant fluid will be collected in sterilized tube.

3.3.3. Egg Inoculation

3.3.3.1. Candling of Eggs

- i. Perform candling of the chicken embryonated eggs of 10 days. Check the viability of the embryos. If no movement seen in the egg, discard.
- ii. Fertile, chicken eggs are transferred to a biological safety cabinet (II) for inoculation.

3.3.3.2. Inoculation of Eggs:

- i. The inoculation site of each egg is wiped with 70% ethanol and allowed to air dry and a hole then punched in the shell.
- ii. Allow 2 or 3 eggs per sample.
- iii. Draw up the inoculum into a syringe, allowing 0.2 ml per egg.
- iv. The syringe and needle is discarded after each group of eggs is inoculated.
- v. Wipe the inoculation site with 70% ethanol and allow to air dry.
- vi. Seal the hole with molten wax or any other suitable sealant. (UHU liquid gum).
- vii. Transfer the inoculated eggs to an incubator and incubate at 37⁰C for 2-5 days.

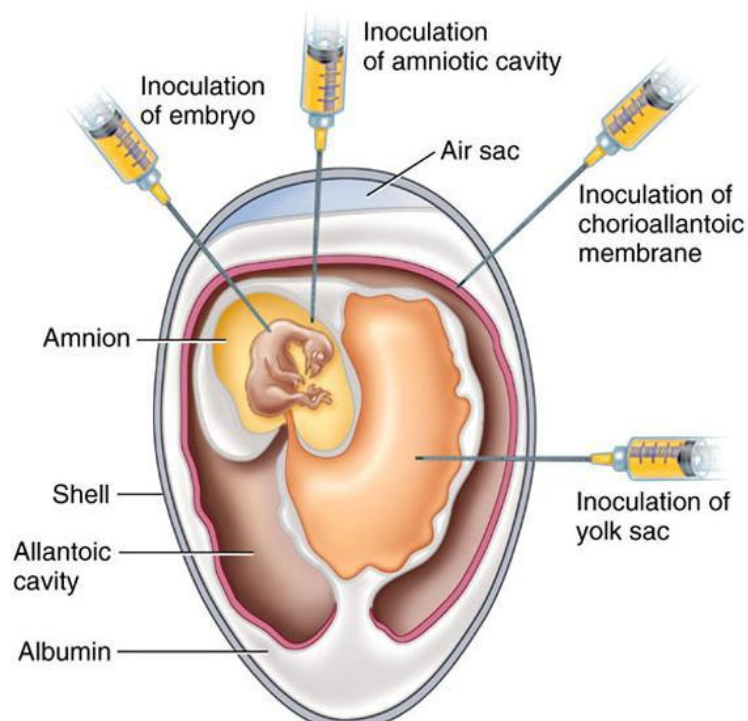


Figure 6: Different routes of inoculation in 10 days chicken embryonated eggs.

3.3.3.3 Harvesting

- i. The eggs are candled daily. Deaths prior to 24 hours post-inoculation are discarded as being non-specific deaths. All deaths after this time are checked for the presence of hemagglutinating agents. If all eggs from any one group are dead within 24 hours the inoculum should be filtered through a 0.2 μ m filter to remove contaminating bacteria and fresh eggs inoculated.
- ii. Surviving eggs are placed at 4⁰C overnight after 5 days of incubation. Eggs may be rapidly chilled at -20⁰C for 2 hours prior to harvesting.
- iii. Allantoic fluid from each of the surviving eggs is tested for the presence of hemagglutinating agents.
- iv. Hemagglutination negative allantoic fluids from eggs that died during the incubation may be pooled with allantoic fluids from surviving eggs of the same group and 0.2 ml is inoculated into the allantoic sac of fertile eggs for a second passage.
- v. Viruses in samples that are positive for HA should be identified by Hemagglutination inhibition tests using reference antisera.

3.3.3.4. Interpretation

Eggs that die within 24 hours of inoculation are discarded. Eggs that die after this time are tested for the presence of hemagglutinating agents. Eggs that die and do not show hemagglutination are considered to be negative for the presence of hemagglutinating viruses. If, after two passages, the eggs show no hemagglutination the samples are considered to be negative for the presence of hemagglutinating viruses.

3.3.4. Hemagglutination test:

3.3.4.1. Hemagglutination Test (HA)/Antigen titration procedure

- i. Dispense 25µl of PBS (pH 7.2) into each well of a plastic U-bottomed microtiter plate.
- ii. Add 25µl of virus suspension/ allantoic fluid) in the first well of each row
- iii. Make serial, two fold dilutions of the virus suspension from column 1(well 1) to column 12 (well 12) and discard 25µl from the last well (well 12).
- iv. Add a further 25µl of PBS to each well.
- v. Add 25µl of 1% (v/v) chicken RBCs to each well. Cover the plate, mix by tapping the plate gently or put it on shaker and shake for 10 to 15 seconds. Then allow the RBCs to settle for about 40 minutes at room temperature, i.e. about 20°C, or for 30-60 minutes at 4°C, if ambient temperatures are high.
- vi. Run negative control (25µl RBCs + 50µl PBS) and positive control (Reference antigen titration).

3.3.4.2. Interpretation of Results:

The plate for hemagglutination activity was examined. The end point is the highest dilution at which there is complete agglutination. In the absence of hemagglutination, RBCs form compact button at the bottom of the wells. HA is determined by tilting the plate and observing the presence or absence of tear-shaped streaming of the RBCs. The titration should be read to the highest dilution giving complete HA (no streaming or agglutination of red blood cells). This represents 1HA unit (HAU) and can be calculated accurately from the initial range of dilutions.

“An HA unit (1HAU) is defined as the reciprocal of the maximum dilution of virus that causes complete clumping (agglutination) of red blood cells when treated with an equal volume of properly diluted RBC's”.

3.3.5. Molecular detection of avian influenza virus

3.3.5.1. Extraction of viral RNA

3.3.5.2. Preparation of RLT working solution

Twenty µl of Mercapto-ethanol was added with 1 ml of RLT stock solution to make working solution.

3.3.5.3. Preparation of RPE working solution

One part RPE stock solution was added with four parts of pure alcohol to make working solution.

3.3.5.4. RNA extraction

Viral RNA was isolated from swab samples using One step RNeasy Kit QIAGEN (Germany) as recommended by the manufacturer.

1. About 450 µl of RLT working buffer was taken into an micro centrifuge tube tube.
2. Then 250 µl swab sample was added, vortex and incubated for 15 minutes at room temperature.
3. Then 700 µl of 70 % ethanol was added.
4. After mixing, 700 µl mixtures was transferred to an RNeasy spin column placed in a 2 ml collection tube and centrifuged at 10,000 rpm for 30 sec.
5. The flow-through was discarded and again set the spin column in the collection tube.
6. Then rest of the mixture was transferred to the column and centrifuged as above and discarded flow-through.
7. Then 700 µl of RW1 buffer was taken to the column, centrifuged as above and the flow-through was discarded.
8. Then 500 µl of RPE was added into the spin column 7. Then centrifuged for 30 sec at 10000 rpm.
9. Flow-through was discarded and again 500 µl of RPE added and centrifuged for 2 min at 10000 rpm.
10. The spin column placed into a 1.5 ml micro centrifuge tube.
11. About 30-50 µl of RNase free water was added into the centre of the spin column
12. Then centrifuged for 1 min at 10000 rpm and the RNeasy spin column was discarded.
13. Then labeled micro centrifuge tube containing RNA.

14. Stored RNA at -20 or at -70 °C for short term and long term storage, respectively.

3.7.4.5. Preparation of reaction mixture

3.7.4.5.1. Detection of Influenza A (Matrix gene) and H9 subtypes

Reaction mixture preparation for the detection of avian influenza M gene and H9 gene using using Ambion AgPath_ID™ One-step RT-PCR Kit and Qiagen One-step RT-PCR Kit respectively:

Table 2: Master mix preparation.

Master Mix preparation for M gene using Qiagen One-step RT-PCR Kit:	
Component	Volume
Buffer (5 ^X)	5.0 µl
Forward primer 151-f (10 pmol)	1.0 µl
Reverse Primer 638-r (10 pmol)	1.0 µl
dNTP	1.0 µl
Enzyme mix	1.0 µl
H ₂ O	14.0 µl
Total	23.0 µl
RNA Template	2.0 µl
Total	25.0 µl

3.7.4.6. Amplification of extracted viral RNA

1. About 17µl for M gene and 23 µl of master mix for H9 gene was taken into a PCR tube (0.2 ml).
2. Then 8 µl for M gene and 02 µl of extracted viral RNA were taken into the PCR tube containing master mix.
3. The tube was placed into the real time PCR equipment for the detection of influenza A virus and into the thermo-cycler immediately for the amplification of H9 gene after adding the master mixture with the RNA and the cyclic program was resumed.

4. After the program was over PCR product was run in 1- 1.5 % agarose-gel for the detection of H9 subtypes.

3.7.4.7. Thermal profile for RT-PCR

The RT-PCR for virus detection was performed as follows: Reverse transcription at 50°C for 30 min, enzyme inactivation at 95°C for 15 min. PCR for M gene was performed as, initial denaturation for 1 min at 95°C, followed by 35 cycles of amplification, with each cycle consisting of 1 min of denaturation at 94°C, 1 min of annealing at 50°C, and 2 min of extension at 72°C followed by final extension for 7 min at 72°C. For N gene, annealing was performed at 55°C for 30 sec and extension at 72°C for 30 sec and final extension was for 10 min. Other conditions were same as M gene.

Table 3: Temperature profile of RT-PCR.

Steps	M gene		H9 gene	
	Temp.	Time	Temp.	Time
Reverse Transcription	45 °C	10 min	50 °C	30 min
Initial denaturation	95 °C	10 min	95 °C	15 min
45 cycles for M gene and 35 for H9 gene-				
Denaturation	94 °C	10 sec	94 °C	30 sec
Annealing	60 °C	30 sec	55 °C	30 sec
Extension	-	-	72 °C	1 min
Final Extension	-	-	72 °C	10 min

3.7.4.8. Procedure of electrophoresis

PCR products were analyzed by 1-1.5 % Agarose-gel electrophoresis. Briefly;

1. Gel containing tray was assembled with gel comb of appropriate teeth size and number.
2. Then 1 % Agarose solution was prepared in TAE buffer by melting the gel powder with a microwave oven for proper melting.
3. Then 0.4 µl Ethidium bromide (1 mg/ml) was added to 40ml melted agarose to have a final concentration of 0.01 µl /ml.

4. Melted agarose was poured onto the casting tray carefully to avoid bumping and allowed to solidify on the bench.
5. The comb was removed from the gel and hardened gel with its tray was transferred to the electrophoresis tank containing sufficient TAE buffer to cover the gel.
6. A total of 10.0 μ l of PCR product was mixed with 1 μ l (5X) loading dye and the sample was loaded to appropriate well of the gel carefully.
7. A total of 0.3 μ l of 100 bp DNA marker was loaded on the one (middle) side of the gel.
8. The lid of the electrophoresis apparatus was connected to power supply and electrophoresis was run at 100 Volt for 35 minutes.
9. When DNA migration was sufficient, as just from the migration of bromophenol blue of loading buffer, the power supply of the apparatus was switched off.
10. Then UV light of the apparatus was switched on; the image of the desired DNA band on the gel was viewed on the monitor and saved on the floppy disc for taking photos.



Figure 7: 1.5% Agarose-gel preparation



Fig 8: Real time PCR machine.

CHAPTER 4

RESULTS

4.1 Results of Hemagglutination Inhibition test

A total of 800 samples among them 500 from migratory birds, 70 from ducks, 190 from commercial poultry and 40 from backyard poultry were collected and processed. Then supernatant fluid were inoculated into 10 days old chicken embryo at yolk sac route and incubated at 37⁰C and checked every day until death of the embryo. In case of embryo mortality, yolk sac fluids were collected on post mortem. If no mortality occur at first passage, all embryos were killed at 14 days post inoculation and allantoic fluid were collected and inoculated for second passage. If no mortality occurs, the sample were discarded and treated as negative. Allantoic fluids collected from dead embryos were subjected to Hemagglutination Inhibition (HI) test. Among all these samples 20 samples from migratory birds, 04 from ducks, 12 from commercial poultry and 02 samples from backyard poultry was Hemagglutination Inhibition (HI) positive. The HI positive sample was further tested by the rRT-PCR and RT-PCR for confirmation of influenza A by detecting Matrix gene.

All the positive samples were sent to the *FAO, OIE and National Reference Laboratory for Avian Influenza and Newcastle Disease* to do further test and confirmation. RRT PCR test was again done to detect for Matrix gene, H5, H7, N9 gene.

4.2 Detection of AI virus by RT PCR:

Avian influenza virus RNA was detected from allantoic fluid of dead embryos which was positive to HI test using standard protocols. RNA was extracted from the samples using Qiagen RNeasy Mini Kit. RT-PCR was performed using AI specific primer sets for Matrix gene from published literature to amplify Matrix gene segments of AI. The RT-PCR product was visualized in 1.5% agarose gel stained with Ethidium bromide. Among 36 HI positive samples, 08 migratory bird samples were positive and 04 commercial poultry samples were positive. Out of

these positive samples only one migratory bird sample was positive for H5N1. Rest of the samples was positive for H9N2. The H5N1-subtype isolate exhibit a polybasic cleavage site (PQRERRRKR*GLF) that is characteristic of highly pathogenic viruses.

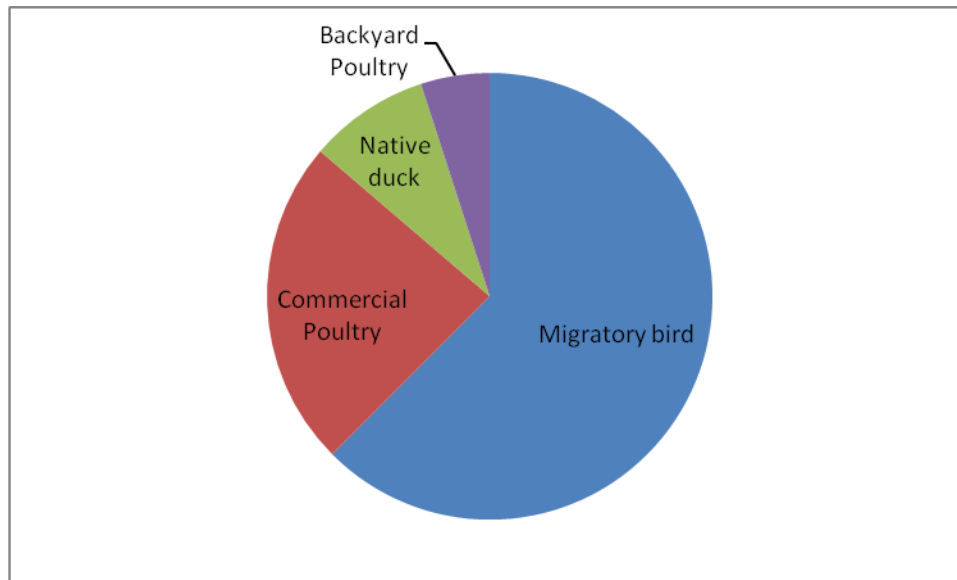


Fig 9: Sampling distribution of present study.

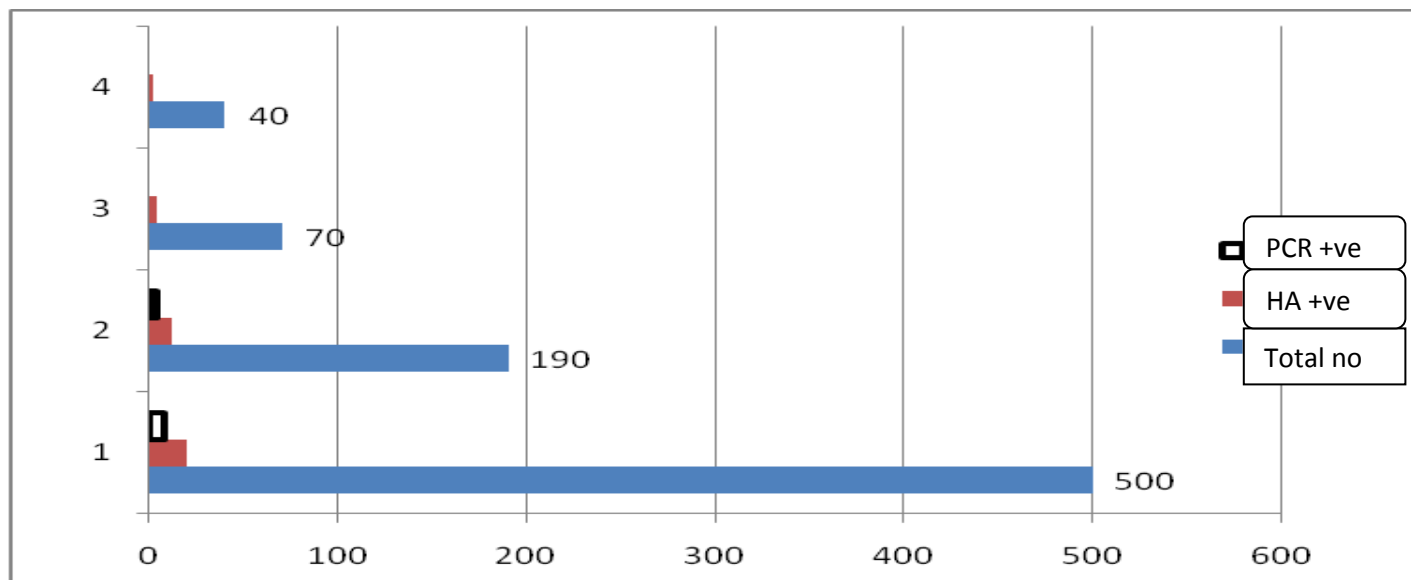


Fig 10: Total, HA and PCR positive sample.

Table 4: AI Surveillance in Dhaka district, Bangladesh.

Type of Sample	Sample No.	HI positive	HI positive (%)	PCR (+Ve)	PCR (+Ve) (%)
Migratory bird	500	20	4.00	8	1.6%
Duck	70	4	5.71	0	0%
LBM	190	12	6.31%	4	2.11%
Backyard poultry	40	2	5.0%	0	0%
Total	800	36	4.50%	12	1.5%

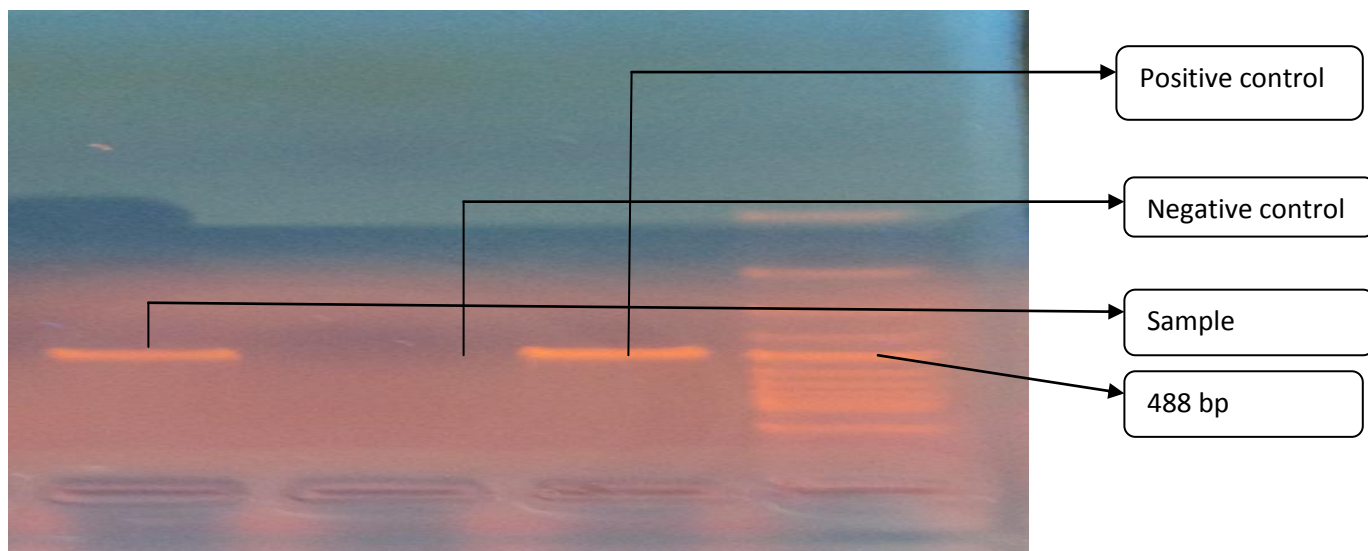


Figure 11: Detection of H9 subtypes of AI virus.



Fig 12: Detection of matrix gene of Avian Influenza.

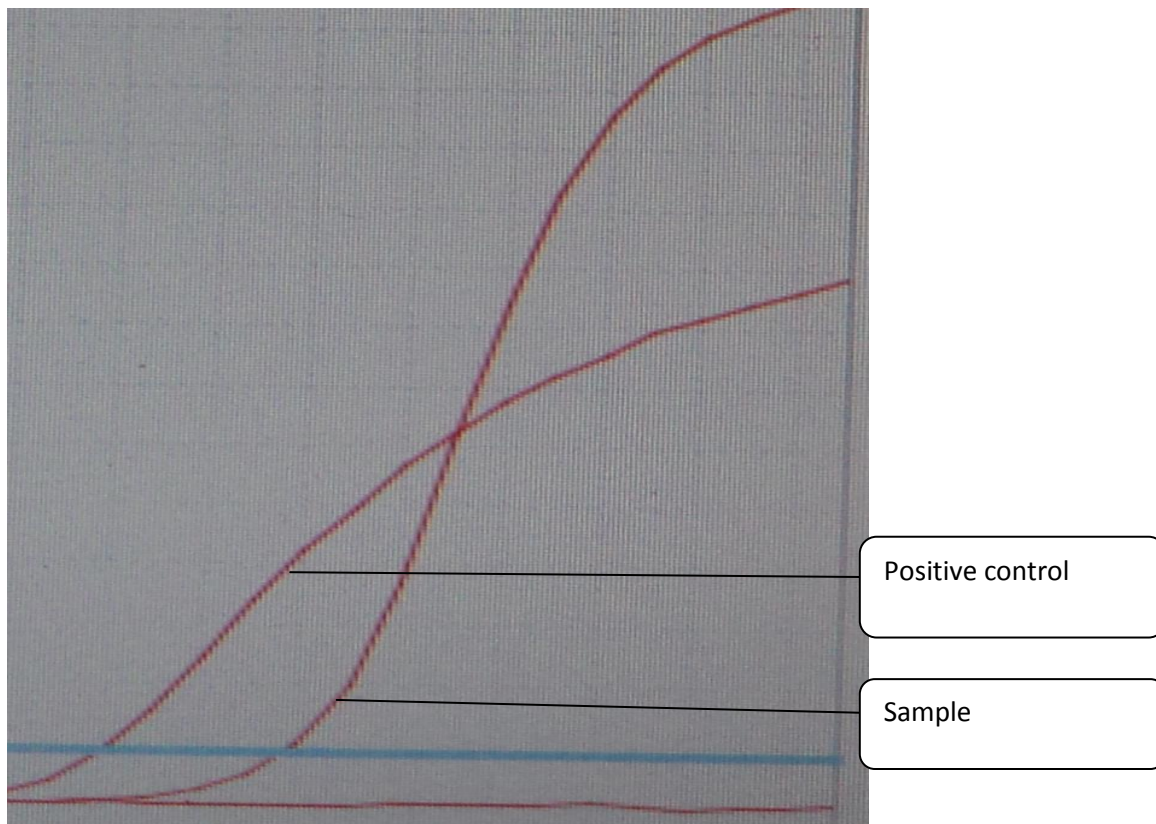


Fig 13: H5 Amplification plot.

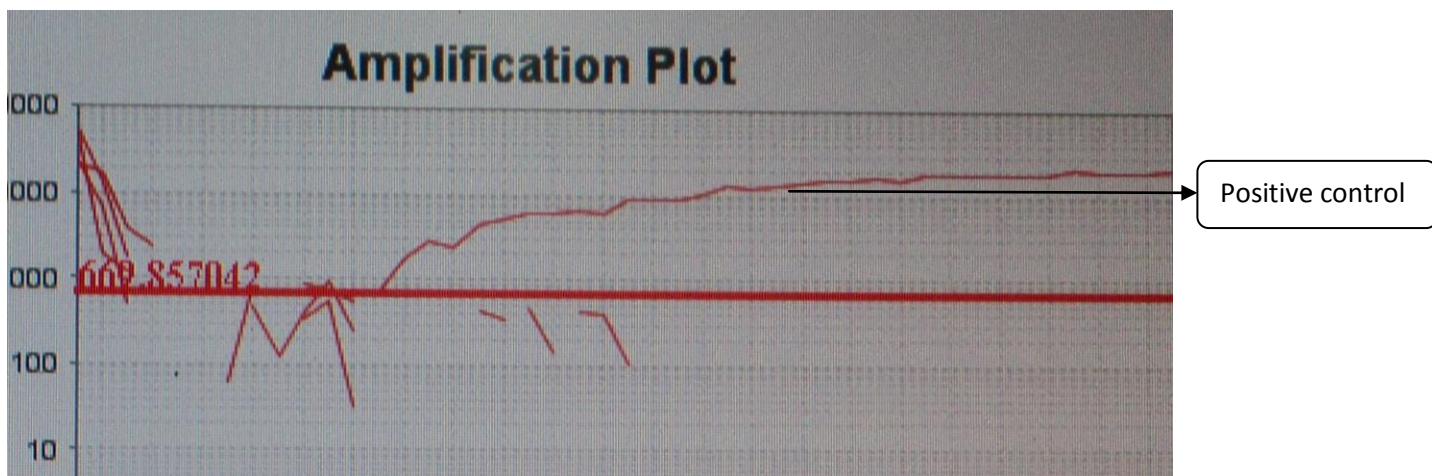


Fig 14: H7 amplification plot.

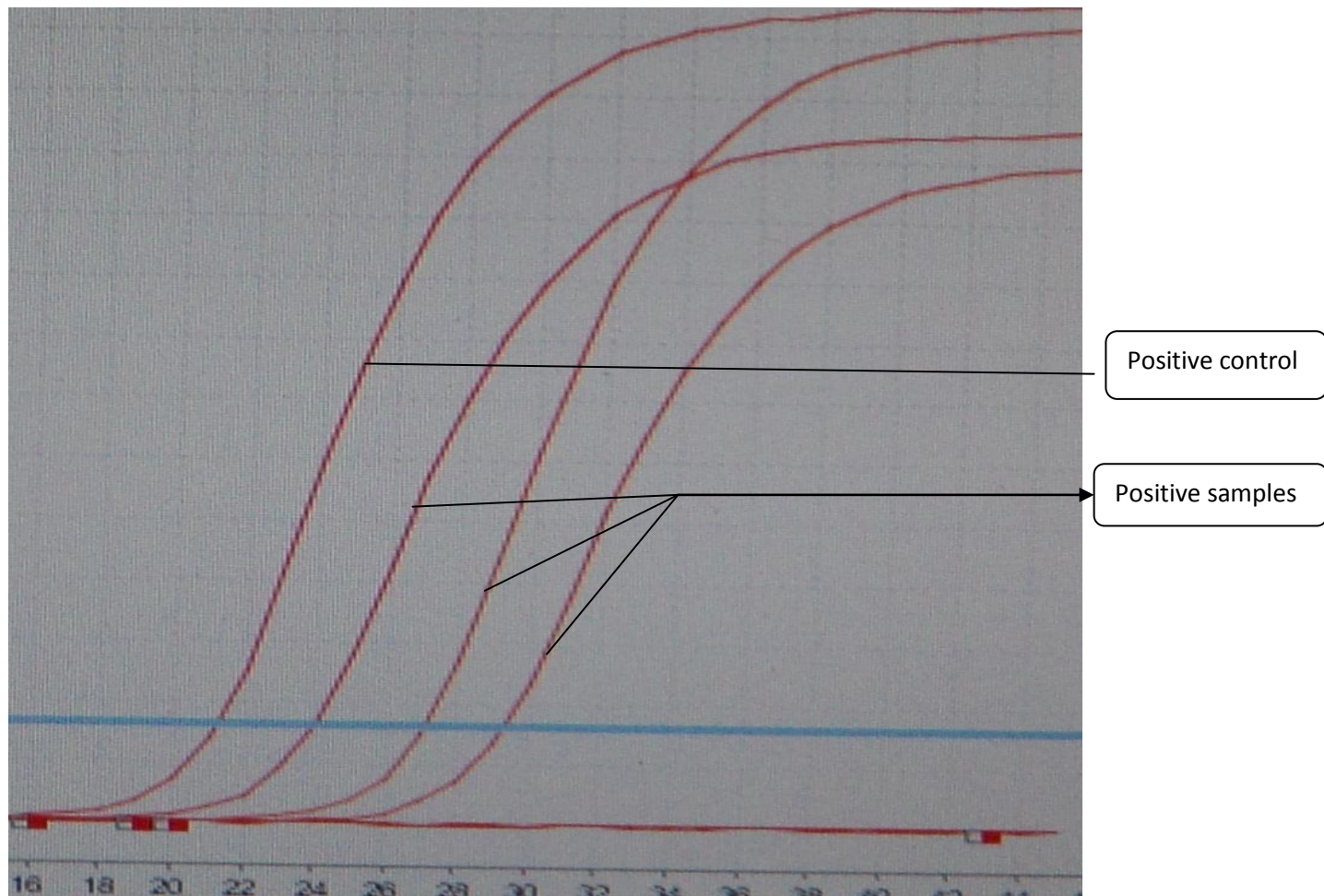


Fig 15: H9 amplification plot

Table 5: Real time PCR result of samples collected from migratory and commercial birds
(Analysis done at FAO, Rome & OIE, Paris).

Bird type	RRT for AI	RRT for H5	RRT for H9	RRT for H7
Migratory (2665/26)	P	P (PQRERRRKR*GLF)	N	N
Migratory (2665/27)	P	N	N	N
Migratory (2665/28)	P	N	N	N
Migratory (2665/29)	P	N	P	N
Migratory (2665/30)	P	N	P	N
LBM (2665/31)	P	N	P	N
LBM (2665/32)	P	N	P	N
LBM (2665/33)	P	N	P	N
LBM (2665/34)	P	N	P	N
Migratory (1121/51)	P	N	N	N
Migratory (1121/52)	P	N	N	N
Migratory (1121/53)	P	N	N	N

CHAPTER 5

DISCUSSION

Bangladesh first experienced highly pathogenic avian influenza (HPAI) in March, 2007 and the National Reference Laboratory for Avian Influenza (NRL-AI) at Bangladesh Livestock Research Laboratory (BLRI) diagnosed and confirmed the presence of H5 sub-type virus. Gene sequencing was done from the isolates diagnosed in 2007-2008 and demonstrated that 99.1 to 100% were of identical genetic structure, which clearly indicates that viral introduction was only once in Bangladesh (Alam J et al, 2010).

Phylogenetic analysis done on Bangladeshi isolates also revealed that isolates belong to the sub-clade 2.2 popularly known as Qinghai lineage or Euro-Asian lineage with highest similarities to those from Kuwait, Mongolia, Russia and Afghanistan (Shanmuganatham K et al, 2013). The close similarities between HPAI isolates of these countries with Bangladesh isolates suggest that migratory birds might be responsible for the initial introduction of HPAI in Bangladesh as the country has no poultry trade link with these countries.

In the time frame of 2007-2014 546 outbreaks have been reported to the OIE (Osmani et al, 2014). Nevertheless Bangladesh was considered free from human infection up to May, 2008. However one human case was announced by the authorities on May 22, 2008. The case was then confirmed by Center for Disease Control and Prevention (CDC) in Atlanta, USA. With this case, Bangladesh has become the 15th country of the world to report human infection with HPAI H5N1 virus (Brooks WA, 2009).

From March 2007 until the end of 2010, the circulating HPAI H5N1 viruses in Bangladesh were all from clade 2.2, sub-lineage III; however, at the beginning of 2011, new introduction of clades 2.3.2.1 and 2.3.4.2 were detected (Islam MR) and this confirms that there were repeated introductions of H5N1 virus into Bangladesh since 2007 (Marinova-Petkova, 2013). Source of repeated introductions are suggested as migratory birds and indicated the need for monitoring the

wild, migratory birds and poultry population as Bangladesh is a tropical country and an important place for migration and over wintering of wild migratory birds.

This present research was under taken with a view to investigate the epidemiological and molecular study of Low pathogenic Avian Influenza (LPAI). Previous studies have shown that low pathogenic avian influenza was found in the clinically healthy population of poultry before the highly pathogenic avian influenza epidemic starts (Alam J et al, 2003).

Several observations were made in this study. As follows:

First, in this study we have found differential avian influenza infection status in different types of birds based on their habitat. No avian influenza infection was found in backyard or house hold poultry and native duck. Highest infection rate found in migratory bird followed by commercial poultry samples collected from live bird market.

Second, we have found 36 HI positive samples; out of which only 12 were PCR positive. This suggests presence of some other viral infection like Newcastle disease which is also HI positive. This issue should be further investigated.

Third, in our study we have found that positive samples in migratory birds are more than those in commercial poultry. Only one HPAI was detected in migratory bird sample collection in winter season. This might suggest that presently migratory birds are carrying more avian virus than they are transmitting. This might suggest a future epidemic. Another explanation is improved biosecurity measures taken on poultry business and increased consciousness among the businessmen due to repeated epidemic. Osmani et al, (2010, 2011) have also predicted that increased enforcement of biosecurity, stronger control of poultry movements, better surveillance and reporting might decrease the outbreak. So on this background the relative contribution of trade and the market chain versus wild birds in spreading the disease needs further investigation.

Fourth, in our study we have also found that no backyard poultry are infected. Much less infection rate in backyard poultry than commercial poultry can be explained by the fact that commercial poultry live in high density area that contains variety of hosts. Also commercial bird comes from different area and infection transmits from healthy to infected bird and that makes live bird market the ideal environment for the influenza transmission. Our backyard poultry sample size was small and was collected sample from three villages.

Fifth, previously done research by khatun et al (2013) found that prevalence of avian influenza in semi-scavenging ducks was 22.05% and they speculated that these ducks might be source of avian influenza infection in commercial backyard poultry. In contrast our finding showed total absence of avian influenza in duck population. The most plausible explanation behind this finding might be the source of sample population. They collected sample from semi-scavenging duck whereas in our study sample was collected from live market bird duck. Most of this live market bird ducks are not semi-scavenging rather they are reared commercially like poultry. Another explanation is that they conducted their study in three consecutive winter season whether our study has been conducted in only one winter season. So the variation of avian influenza prevalence according to season or year might be a crucial factor here. Moreover our sample size was also much smaller than the previously reported study.

Because of legal restrictions imparted by Government of Bangladesh, limited logistics support and difficulties in trapping of the migratory and wild birds, larger sample sizes of cloacal, tracheal swabs and blood samples are very difficult to collect. For these reasons our collected and screened samples only include environmental sample. These samples have decreased sensitivity.

CHAPTER 6

CONCLUSIONS AND RECOMMENDATIONS

Influenza virus is a negative sense single stranded RNA virus in Orthomyxoviridae family and the causative agent of avian influenza; an acute viral disease of wide spectrum of animal which includes birds, pigs and also human. The disease causes severe losses to poultry production and is presently considered as one of the major threats to millions of poultry population of Bangladesh where mortality may reach up to 100 % in an outbreak. Avian influenza was first detected in Bangladesh in 2007 and since then more than 500 outbreaks has occurred in past years. Although there is a substantial body of literature relating to the epidemiology of influenza viruses in both humans and animals, it is important to review the disease situation, surveillance, and control strategies from time to time. Updated information can be used to direct future research into AI epidemiology and devise more effective programmes to control the disease. Moreover very limited studies have been done on the prevalence of Low pathogenic avian influenza in concurrently in wild migratory birds, commercial and backyard poultry and native duck.

Peiris *et al.* (2007) stated the H5N1 HPAI virus that evolved and resulted in the massive epizootic from 2003 to the present, not only resulted in fatalities in both wild and domesticated birds, but also caused disease with a high mortality rate in humans and other mammals. Cinatl *et al.* (2007) also stated that mutations may also occur at any time which could result in a human to human transmissible strain developing. So eradicating avian influenza not only will secure our poultry sector but also prevent future threat of epidemic.

In conclusion, mixing of different species is thought to increase the chance of reassortment and interspecies transmission of AI viruses. It remains difficult to predict further virus evolution, so strategies are required to prevent the evolution of AI viruses and the emergence of pandemics. Proposed measures include the separation of species, increased biosecurity, better understanding of the virus, and improved vaccination strategies.

Recommendations:

Based on the findings of the present study, we have assumed that further study are to be conducted to know detail about the seasonal effect on disease, environmental factors contribute to the occurrence of the disease, host-virus interaction and molecular properties of currently circulating strain of avian influenza virus in Bangladesh. The following recommendations are made for future studies on Avian Influenza Virus:

1. Large scale surveillance study for the presence of Avian influenza.
2. Detail molecular epidemiology of the virus
3. Avian influenza virus whole genome sequencing.
4. Determination of molecular similarity and dissimilarity between virus circulating and isolated from migratory bird, commercial poultry, backyard poultry and duck.
5. Determination of sensitivity of different host system for virus in a large scale.
6. Determination molecular reason behind pathogenecity.
7. With the availability of the complete genetic information on the virus, research on both vaccine development and therapeutic intervention may significantly contribute to effective disease management in birds helping to reduce the incidence of the disease nationally, regionally as well as globally.
8. All these steps will ultimately lead to eradication of Avian influenza from Bangladesh as many developed did already.

Chapter 7

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