PATTERNS OF AVIAN INFLUENZA VIRUSES CIRCULATION IN POULTRY SOLD AT LIVE BIRD MARKETS AND FACTORS ASSOCIATED WITH THE RISK OF AVIAN INFLUENZA VIRUSES IN BANGLADESH

Sukanta Chowdhury

Registration No. 0006

Session: 2014-2015

A dissertation submitted in the total fulfillment of the requirements for the degree of Doctor of Philosophy in Epidemiology

Department of Medicine and Surgery

Faculty of Veterinary Medicine

Chittagong Veterinary and Animal Sciences University

Chittagong-4225, Bangladesh

January 2019

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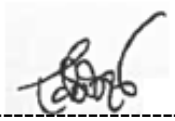
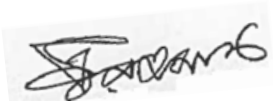


Supervisor
Md. Ahasanul Hoque, PhD
Professor of Veterinary Epidemiology
Department of Medicine and Surgery
Chittagong Veterinary and Animal Sciences University

Co-Supervisor
Erin D. Kennedy, DVM, MS, MPH
Center for Global Health
Centers for Disease Control and Prevention
Atlanta, USA



Chairman of the Examination Committee
Prof. Md. Mizanur Rahman, PhD
Department of Medicine and Surgery
Chittagong Veterinary and Animal Sciences University

Department of Medicine and Surgery
Faculty of Veterinary Medicine
Chittagong Veterinary and Animal Sciences University
Chittagong-4225, Bangladesh

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List of Abbreviations

Abbreviation	Elaboration
AI	Avian Influenza
AIV	Avian Influenza Virus
AEEC	Animal Experimentation Ethics Committee
AVS	Additional Veterinary Surgeons
APR	Adjusted Prevalence Ratio
BBS	Bangladesh Bureau of Statistics
BMD	Bangladesh Meteorological Department
BSL	Biosafety Level
CC	Communication Committee
CDC	Centers for Disease Control and Prevention
CAHW	Community Animal Health Worker
CI	Confidence Interval
CVASU	Chittagong Veterinary and Animal Sciences University
^{o}C	Degree Centigrade
DLS	Department of Livestock Services
DMCC	District Multi-sectoral Co-ordination Committee
DOC	Day-old Chick
DVM	Doctor of Veterinary Medicine
eg	For Example
etc.	Et cetera
FAO	Food and Agriculture Organization
GISAID	Global Initiative on Sharing All Influenza Data
GoB	Government of Bangladesh
HA	Hemagglutinin
HI	Hemagglutination Inhibition
HPAI	Highly Pathogenic Avian Influenza
HPAIV	Highly Pathogenic Avian Influenza Virus
icddr,b	International Centre for Diarrhoeal Disease Research, Bangladesh
i.e	That is
IEDCR	Institute of Epidemiology, Disease Control and Research
JTC	Joint Technical Committee
LBM	Live Bird Market
LPAI	Low Pathogenic Avian Influenza
LPAIV	Low Pathogenic Avian Influenza Virus

M	Matrix
M-gene	Matrix Gene
MoHFW	Minister of Ministry of Health and Family Welfare
MSc	Masters of Science
MDCK	Madin Darby Canine Kidney
n	Number
NA	Neuraminidase
NDVI	Normalized Difference Vegetation Index
NRRT	National Rapid Response Team
NMTF	National Multi-sectoral Task Force
NAC	National Advisory Committee
NP	Nucleoprotein
OIE	Office International des Epizooties (World Organization for Animal Health)
OR	Odds Ratio
p	Probability
PAF	Periodic Annual Function
PCR	Polymerase Chain Reaction
PhD	Doctor of Philosophy
PBS	Phosphate Buffered Saline
PR	Prevalence Ratio
r	Correlation coefficient
RNA	Ribonucleic Acid
rRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
SA	Sialic Acid
SMS	Short Message Service
UNICEF	United Nations International Children's Emergency Fund
ULO	Upazilla Livestock Officer
UMCC	Upazila Multi-sectoral Co-ordination Committee
URT	Upper Respiratory Tract
USA	United States of America
USAID	United States Agency for International Development
USD	United States Dollar
VTM	Viral Transport Medium
WB	World Bank
WHO	World Health Organization

Table of Contents

List of Figures

List of Tables

Abstract

H5N1 virus has caused repeated outbreaks in the poultry sector in Bangladesh. More than 550 reported HPAI H5N1 outbreaks in poultry and eight human cases, including one death, have been reported since 2007. In Bangladesh, multiple poultry species including domestic chickens, ducks, geese, pigeon and quail are reared together in backyard farms and sold in live bird market (LBM), facilitating avian influenza viral transmission between species. Though many epidemiological studies from Bangladesh have been conducted to detect avian influenza viruses (AIVs) in poultry, long term surveillance data on avian influenza prevalence are more useful and effective for local preparedness and control activities. We have been conducting LBM based surveillance since 2007 to evaluate AIVs circulation among poultry reared in different systems in Bangladesh. Here, we described the pattern of avian viruses circulation among domestic poultry from August 2007 to December 2016. A pair wise Spearman correlation coefficient was calculated to identify the role of climatic factors on the occurrence of AI. Another study was conducted to identify the association between biosecurity practices and environmental contamination with AI viruses.

Over the surveillance period (2007-2016), Avian influenza (AI) A viral RNA was detected in 6% (95% CI: 5.7-6.8) waterfowl, 3% (95% CI: 2.9-3.9) commercial chicken, 2% (95% CI: 1.2-2.5) backyard chicken and 29% (95% CI: 27.2-31.3) environmental samples. Subtypes H5N1, H9N2, H11N3, H4N6 and H1N1 viruses were commonly detected. We detected 5 clades for H5N1 viruses; clade 2.2, 2.3.2, 2.3.4, 2.3.2.1 and 2.3.2.1a. Among the all detected clades, clade 2.3.2.1 was the predominant one, circulating since 2011. Among the waterfowl, ducks were more likely to be positive for AI A viruses compared to geese (OR 3.6, 95% CI: 2.3-5.7). The domestic waterfowl which were sampled during winter season were more likely to be tested positive for influenza A viruses compared to the waterfowl sampled during summer and monsoon (OR 2.4, 95% CI: 1.9-3.1). Among commercial chicken, Cobb type broiler chicken were more likely to be positive for AI A viruses than broiler, layer and breeder (OR 9.8, 95% CI: 3.1-31.1). Environmental samples collected from urban LBMs were more positive for AI A/H5 than the rural or peri-urban LBMs specimens (OR 2.2, 95% CI: 1.7-2.8).

In waterfowl, there was a single annual peak of AIV occurrence that mostly observed in between December and January across different years. No clear seasonal pattern of AIV occurrence was observed in commercial and backyard chicken. However, there were two periodic annual signals of AIV occurrence were pronounced in environmental samples (September and February). In waterfowl, AIV circulation was negatively correlated with the climate variables (monthly average temperature, average humidity, total precipitation and average wind speed). In commercial chicken, AIVs circulation was positively correlated with humidity and precipitation, but negatively correlated with temperature and wind speed. In backyard chicken, AIV circulation was negatively correlated with all climate variables except wind speed.

Poultry shops that slaughtered poultry within their shops (APR 1.6, CI: 1.1-2.3) and/or shops with unsold poultry from the previous day (APR 1.9, CI: 1.3-2.8) and/or absence of a weekly rest day (APR 1.2, CI: 1.1-1.4) and/or keep sick and healthy poultry together (APR 1.2, CI: 1.1-1.4) were more likely to harbor detectable AIV RNA.

The findings of the surveillance suggest that avian influenza A viruses, including H5 and H9 subtypes, circulate year-round in poultry in Bangladesh. AI seasonality study suggest that circulation of AI was seasonal only in waterfowl. Avian influenza viruses were circulating in commercial chicken throughout the year indicating AI is endemic among commercial chicken in Bangladesh. Existing cleaning and disinfection practices were not significantly associated with decreased environmental contamination with AIVs. LBM based active surveillance provided valuable information about the status of AIVs in poultry in Bangladesh. Findings from molecular analysis will help public health policy makers develop and update candidate vaccine viruses for pandemic preparedness. Identification of certain risky biosecurity practices such as poultry slaughtering practices, management of leftover poultry, weekly rest day, cleaning and disinfection is necessary for improvement. Overall, this research will improve our understanding about AIVs dynamics and also help us develop intervention strategies for preventing and controlling AI in poultry and humans in Bangladesh.

Keywords**:** Avian Influenza, Poultry, Seasonality, Climate Factors, Live Bird Market, Environmental Contamination, Biosecurity, Bangladesh

Chapter-1: General Introduction

A comprehensive literature review was performed based on previously published and un-published articles or reports on the epidemiology of avian influenza (AI) at animal-human interfaces. This literature review helped identify the scientific gaps that influenced the need to conduct the PhD research on dynamic patterns of AI circulation in poultry sold at Live Bird Markets (LBMs) and factors associated with the risk of AI circulation in Bangladesh. The areas of literature reviewed included AI and its transmission and associated risk factors, prevalence of AI and its sub-type distribution in poultry, outbreaks of AI in poultry and molecular status, the role of LBMs in the epidemiology of AI, occurrence of human cases with highly pathogenic avian influenza (HPAI), AI surveillance systems in Bangladesh, national AI preparedness and response plan, effects of climate factors on the incidence of AI, economic and public health consequence, diagnosis of AI, and prevention and control.

1.1. Introduction

Highly Pathogenic Avian Influenza has caused a large number of outbreaks in different poultry sectors in Asia, Europe, and Africa (OIE, 2018: http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2016/). H5 and H7 subtypes of influenza type A virus are known to be highly pathogenic in gallinaceous poultry. H5 and H7 subtypes can mutate spontaneously at HA cleavage site to become highly pathogenic to poultry (Taubenberger and Morens, 2017). Avian influenza viruses (AIVs) have a diversified host range and can cross the species barrier. Avian influenza hosts include waterfowl, terrestrial and aquatic poultry, swine, humans, horses, dogs, cats, whales, seals and several other mammalian species (Short et al., 2015). Wild aquatic birds (shorebirds, gulls and ducks) are considered as natural reservoir hosts for AI. They carry and shed HPAI viruses without showing any clinical manifestation (Ellis et al., 2004; Hulse-Post et al., 2005). Chickens are the most susceptible poultry species to HPAI H5N1 with high morbidity and a case fatality rate as high as 100% (Alexander, 2007). After 1996, HPAI H5N1 reemerged in 2003 in both poultry and humans of Southeast Asian countries (OIE, 2018; WHO, 2018).

1.1.1. Avian Influenza

Influenza viruses are pleomorphic, negative sense, single-stranded, segmented and enveloped RNA viruses belonging to the family of *Orthomyxoviridae*. The segmented genome consists of 8 single-stranded, negative sense RNA molecules that encode 10 proteins. They have two distinct glycoproteins: the haemagglutinin (HA) and neuraminidase (NA). Haemagglutinin is the key determinant to which neutralizing antibodies are directed and NA is the second important determinant for neutralizing antibodies. A membrane protein known as Matrix 2 (M2) protein is present in small quantities. The RNA segment remains within the viral envelop in association with the nucleoprotein (NP) and three subunits of viral polymerase (Polymerase Acidic: PA, Polymerase Basic 1: PB1 and Polymerase Basic 2: PB2). Based on antigenic differences in NP and M protein, influenza viruses are classified into three types; A, B and C (de Jong and Hien, 2006).

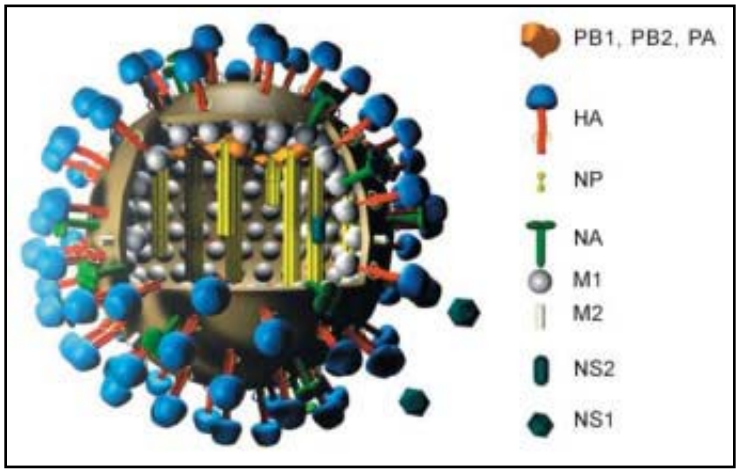


Figure 1.1. Structure of an influenza A virus (Gürtler 2006)

Based on HA and NA surface proteins, influenza A viruses are classified according to subtypes. The HA and NA genes vary in sequence, a total of 18 HA and 11 NA subtypes were identified (Fouchier et al., 2005; Schrauwen and Fouchier 2014). The HA glycoprotein plays an important role in determining the host range of influenza viruses. Haemagglutinin proteins have different receptor specificities and they can recognize oligosaccharides containing terminal sialic acid on the surface of epithelial cells (Cauldwell et al., 2014). The NA glycoprotein removes sialic acid

residues from cellular receptors and extracellular inhibitors that facilitate the mobility of virions and their release from cells. For virus replication and transmission, an optimal balance between HA and NA is required. Viral reassortment and transmission to new hosts depends on the balance of HA and NA (Cauldwell et al., 2014).

The HA is the attachment protein that binds with sialic acid (SA) sugar, which acts as receptors on host cell surface. The efficient binding between attachment protein and the host cell requires abundant receptors in the upper respiratory tract (URT) of humans for virus transmission. Most natural strains of AIV do not bind well enough to the human receptors to be infectious at the low doses (Matrosovich et al., 2009).

The human glycome (repertoire of glycan sugars) differs from chickens and aquatic birds. α 2,6 linked SA receptors are abundant in the URT of humans. Both α 2,6- and α 2,3-linked SAs are present in the chicken nasal cavity, URT, and gut, whereas α 2,3-linked SA is predominant in ducks (Fig. 1.2) (Gambaryan et al., 2002; Kuchipudi et al., 2009; Costa et al., 2012) .

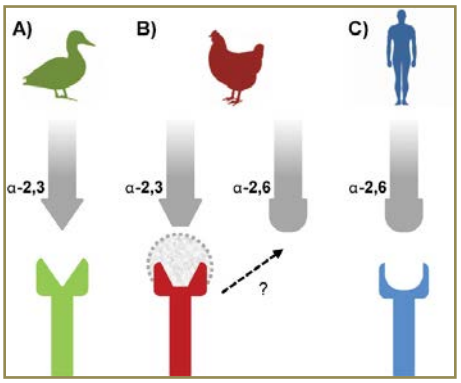


Figure 1.2. Differences in the glycome of influenza hosts drive changes in influenza HA (Long et al., 2015)

1.1.2. Transmission of Avian Influenza

Avian influenza viruses transmit among birds through fecal-contaminated aerosols, water and feed. The fecal-oral route via surface water was suspected for aquatic birds (Webster et al., 1978; Webster et al., 1992).

Exposure to AIVs (including H5N1) through contact with infected blood or bodily fluids of infected poultry, touching or caring for infected poultry, consuming uncooked and under cooked poultry products, swimming or bathing in potentially virus laden ponds and visiting LBMs were identified as common exposure which facilitate the transmission of AIVs to human (Chotpitayasunondh et al., 2005; Van Kerkhove et al., 2011).

Although transmission of influenza virus between humans has been reported to occur through inhalation of respiratory droplets or aerosols containing infectious virus (Tellier, 2006; Cowling et al., 2013; Killingley and Nguyen-Van-Tam, 2013), HPAI H5N1 transmission from human to human is rare or self sustained (Wang et al., 2008).

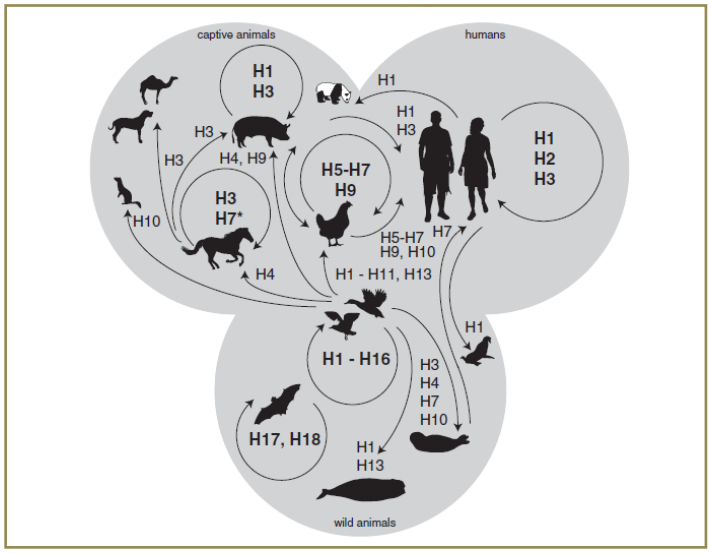


Figure 1.3. Inter-species transmission cycle of avian influenza (Short et al., 2015)

1.1.3. Risk Factors Associated with Avian Influenza

In Vietnam and Indonesia, ducks frequently visited wetlands that are used for rice production, which could be a risk factor for HPAI persistence and spread to other poultry (Gilbert et al., 2006; Henning et al., 2010). In the Netherlands, layer finisher type poultry were more at risk for HPAI outbreak than other types poultry (Thomas et al., 2005). In South Africa, ostrich farms with poor cleaning of food trough (OR 4.5, 95% CI: 1.5-13.3) and failure to clean and disinfect (OR 2.3, 95% CI: 1.1-4.8) were more likely to increase the risk for AI infection (Thompson et al., 2008).

In Vietnam, poultry that received an AI vaccine were less likely to be infected with H5N1 than non-vaccinated poultry. The presence of geese on farms, sharing of scavenging areas with ducks from other farms and presence of visitors in the farm increased the risk of HPAI H5N1 outbreaks in poultry flocks in Indonesia (Henning et al., 2009).

In Southern Europe, backyard poultry raised in free-range farming systems are at high risk for AIV introduction from wild waterfowl (Terregino et al., 2007).

In Japan, chicken farms that introduced end-of-lay chickens; shared farm equipment among farms; practiced incomplete hygiene measures of farm visitors on shoes, clothes and hands; and had direct distance to the nearest case farm were more likely to be case farms for low pathogenic H5N2 virus (Nishiguchi et al., 2007).

In Bangladesh, egg trays and vehicles from local bird markets could be the source of infection in backyard chicken (Biswas et al., 2008). Offering slaughter remnants from purchased chickens to backyard chickens and having a nearby water body were determined as risk factors for HPAI H5N1 in backyard chicken (Biswas et al., 2009b). Human population density, commercial poultry population and the number of roads per sub-district were associated with the occurrence of HPAI H5N1 in commercial poultry (Loth et al., 2010). Numbers of staff, frequency of veterinary visits, presence of village chickens roaming on the farm and staff trading birds were determined as risk factors for HPAI H5N1 outbreaks (Osmani et al., 2014a). Commercial farms that were accessible to feral and wild animals were more likely to be infected with H5N1 (Biswas et al., 2009a).

Live bird markets can play an important role for AIV transmission among avian species and humans (Shortridge et al., 1998). A study from Vietnam provided evidence that retail market were more likely to be the source of poultry with H5N1 outbreak in farms than wholesale markets (Magalhães et al., 2010). This high risk could be due the shorter travel distance between retail markets and communities compared to the distance between wholesale markets and communities.

Slaughtering poultry within LBM and handling of sick poultry have been reported as risk factors for AI transmission to poultry and humans at LBMs (Webster et al., 2004; Indriani et al., 2010).

Highly Pathogenic Avian Influenza H5N1 has been identified from specimens obtained from live bird and food markets during winter (Nov-Jan) in Thailand and interestingly the virus was identified or detected from meat samples of quail, hen and water cock (Amonsin et al., 2008). It is also reported that most of LBM poultry in Thailand came from backyard farms, where they were in unavoidably close contact with wild birds and HPAI H5N1 was also detected in wild birds (such as sparrow) in Thailand (Amonsin et al., 2008).

Forty seven percent of Indonesia LBMs had environmental contamination with AI where slaughtering birds within LBM was identified as a significant risk factor (Indriani et al., 2010).

Avian influenza A (H5N6) virus was detected in air samples (7.8% AIV RNA positive) of LBM in China and these findings indicated that AI in the aerosolized environment of LBM may influence the transmission risk among people in LBM (Wu et al., 2017). BALZAC (Behavioural adaptations in live poultry trading and farming systems and zoonoses control) project also detected AIV and its subtypes in air samples of LBMs in Bangladesh (39% for AI type A, 0.8% for H5 subtype and 21% for H9 subtype (Mahbubur Rahman, personal communication in 2018).

Poultry workers are at high risk of getting H5 infection through infected or carrier poultry exposure during handling, butchering and processing of poultry at LBM (Bridges et al., 2002). In Bangladesh, poultry workers who are involved in feeding poultry, cleaning feces from pens, cleaning food/water containers, and not washing their hands after touching sick poultry were more likely to be at risk for infection compared with workers who infrequently performed these behaviors or practices (RR 7.6, 95% CI: 2.8-20.9) (Nasreen et al., 2015).

Direct involvement with poultry including plucking and preparing of diseased birds, handling fighting cocks, and playing with asymptomatic infected household and market ducks, and consumption of duck's blood or possibly undercooked poultry were identified risk factors for

human infection with HPAI H5N1 (Mounts et al., 1999; Beigel et al., 2005; Ungchusak et al., 2005; Wang et al., 2006).

Although poultry farm level and market level risk factors and other biosecurity practices were studied earlier in Bangladesh (Biswas et al., 2009a; Biswas et al., 2009b; Ahmed et al., 2012; Biswas et al., 2015; Biswas et al., 2011), shop level biosecurity practices in relation to environmental contamination with AI have not comprehensively been studied across the country although studied shop level AI and associated hygienic practices in Chittagong, Bangladesh (Sayeed et al., 2017). A better understanding about shop level bio-security practices is therefore necessary to reduce environmental contamination within LBM. This knowledge gap motivated us to conduct a study on estimating the association between biosecurity practices and environmental contamination with AI in LBMs, Bangladesh.

1.1.4. Avian Influenza Prevalence in Poultry

Many national and international studies reported AI prevalence in different poultry species, with 26 to 63% in commercial layer chicken, 23 to 83% in commercial broiler chicken, 4 to 73% in backyard chickens, 5 to 25% in ducks, 1 to 19% in geese, 1.9% in swan, 71% in apparently healthy broiler breeder, 15% in pigeon and 34% in quail (Naeem et al., 2003; Al-Natour and Abo-Shehada 2005; Olsen et al., 2006; Woo and Park, 2008; Hadipour, 2010; Madsen et al., 2013; Turner et al 2017).

The AI prevalence for avian influenza type A in poultry collected from LBM in Bangladesh was reported to be 23%. Among the influenza-positive specimens, H9N2 subtype was 94% and H5N1 0.08% (Negovetich et al., 2011). The overall prevalence estimate of AI in avian species in LBM in Bangladesh was 24%. Among the influenza-positive specimens, 34% were quail, 25% were ducks, 23% were chickens, , 19% were geese and 15% were pigeon (Turner et al., 2017).

Though many epidemiological studies from Bangladesh have been conducted to detect AIVs in poultry, long term surveillance data on AI prevalence are more useful than small scale studies for local preparedness and control activities. Understanding the circulation of different strains of AI

in poultry populations would provide insight on the transmission process of this virus. We therefore aimed to carry out a LBM-based surveillance study to detect AI in domestic poultry including waterfowl, commercial chicken and backyard chicken.

1.1.5. Avian Influenza Subtype Diversity

Multiple AIV subtypes have been reported in LBM and non LBM premises in the USA such as H1, H2, H3, H4, H6, H7, H9, H10, and H11 (Panigrahy et al., 2002). A multiyear surveillance effort from USA detected AI in 8.5% of avian samples. However, a low level of HPAI viruses including H5 (0.7%), and H7 (0.6%) were detected (Ferro et al., 2010).

Various AI surveillance efforts in different European countries detected multiple AIV subtypes: H1N1, H1N3, H3N8, H4N6, H5N1, H5N2, H5N3, H6N2, H7N4, H7N7, H9N2, H10N4, H10N7, H10N8 and H11N9 in backyard poultry flocks and wild birds in Italy (Terregino et al 2007); LPAI H5 and H7 in wild birds in Denmark and Greenland (Hjulsager et al., 2012); HPAI H7N7 in both backyard poultry and commercial poultry during the outbreak in the Netherlands (Bavinck et al., 2009); and H4, H5 and H6 in Bulgaria. H4 subtype was isolated from mallard ducks and H6 subtype from ducklings in Bulgaria (Goujgoulova and Oreshkova, 2007).

Many AI surveillance efforts in Asian countries reported multiple AIV subtypes such as H7N9 in humans and poultry in 2013 in China (Uyeki and Cox, 2013), H3N2, H4N6, H6N1 and H9N2 in poultry sold at LBM in Korea (Pawar et al., 2012) and HPAI H5N1 and LPAI H9N2 outbreaks in poultry in Pakistan (Naeem et al., 2007). The Chinese H7N9 virus is low pathogenic in wild birds and domestic poultry, but this virus is adapted better than other avian viruses to infect humans (Uyeki and Cox, 2013).

In Bangladesh, multiple subtypes of LPAI along with HPAI H5N1 were detected in different poultry species and in LBM environments. The H1N1, H4N6, H9N2 and H11N3 strains were most frequently identified, whereas H1N3, H2N4, H3N2, H3N6, H3N8, H4N2, H5N2, H6N1, H6N7 and H7N9 were less commonly detected (Gerloff et al., 2016). Another surveillance effort detected H1N2, H1N3, H3N6, H4N2, H5N1, H9N2 and H10N7 subtypes in poultry sold at

LBMs. However, the HPAI H5N1 was detected with extremely low prevalence in poultry sold at market (Negovetich et al., 2011).

A long term active surveillance programme is important to identify both HPAI and LPAI subtypes in poultry. One of the aims of this study was to monitor both HPAI and LPAI among domestic poultry and environments in LBM. This LBM based active surveillance will provide valuable information about the status of AI in poultry in Bangladesh.

1.1.6. Outbreaks of Avian Influenza in Poultry and Molecular status

The first outbreak of HPAI H5N1 was reported in waterfowl in 1996 in China (FAO, 2004a; FAO, 2004b). Since then, fifty six countries have reported HPAI outbreaks in animals, mostly in domestic poultry and wild birds (OIE, 2018). The first HPAI H5N1 outbreaks in different sectors of poultry in south-Asian countries were reported as follows: Indonesia in 2003 (Clade 2), Laos in 2003 (Clade 1), Vietnam in 2003 (Clade 1), Cambodia in 2004 (Clade 1), Thailand in 2004 (Clade 1), India in 2006 (Clade 2.2), Myanmar in 2006 (Clade 7, 2.3.2 and 2.3.4), Pakistan in 2006 (Clade 2.2) and Nepal in 2010 (clade 2.3.2) and (Gutiérrez et al., 2009; Mon et al., 2012; Nagarajan et al., 2012). Pakistan also reported an H9N2 outbreak in poultry sector in 1995 (Abbas et al., 2010). Clades 1 and 2 of H5N1 viruses were circulated in South East Asia between 2003 and 2006. Clade 2 of H5N1 viruses has changed over time, particularly in South East Asia (Gutiérrez et al., 2009). The number of reported HPAI H5N1 outbreaks in different countries is given in appendix I.

After the first HPAI outbreak the subsequent outbreaks occurred in the aforementioned countries. For instance in India, the outbreaks occurred by H5N1 clade 2.2 in 2009 and 2011, and 2.3.2 in 2011 (Tripura, India) (Nagarajan et al., 2012).

In Bangladesh, the first HPAI H5N1 outbreak was officially reported by the government in poultry in March 2007 (Biswas et al., 2008; Loth et al 2010). Since then HPAI H5N1 has caused more than 550 outbreaks in poultry (OIE, 2018). Clades of H5N1 subtypes in these outbreaks have changed over time because of molecular evolution of the virus (such as mutation,

reassortment etc.) (Gerloff et al., 2014). The initial H5N1 clade was 2.2 in chicken (backyard and commercial) (2007), followed by 2.3.4 in commercial chicken (2011), and 2.3.2 and 2.3.2.1 in crow, quail and duck (2011) (Mondal et al., 2013). Clade 2.3.2 was phylogenitically related to newly identified clade 2.3.2.1, recently reported from Asia and Eastern Europe. Clade 2.3.2.1 was primarily isolated from a crow, a quail and a duck (Islam et al., 2012).

In 2011, an unusual mortality occurred in house crows in Bangladesh and HPAI H5N1 clade 2.3.2.1 was determined from the samples of dead crow. During the house crow outbreak, environmental samples were collected from nearby LBMs and the same clade was evidenced, which is suggestive of virus exchange between house crows and market birds (Khan et al., 2014).

The spatial and temporal patterns of HPAI H5N1 outbreaks as well as different Bangladeshi HPAI H5N1 clades are presented in Figures 1.4, 1.5 and 1.6. Two reassortments of H5N1 viruses were detected, carrying the M gene from the Chinese H9N2 lineage. No reassortment was detected between the local H9N2 viruses and H5N1 genotype (Marinova-Petkova et al., 2014). The two human isolates from Bangladesh were closely related to clade 2.2 isolated from Bangladesh chickens indicating that these human cases got the infection from chickens (Hoque et al., 2013; Mondal et al., 2013; Osmani et al., 2014b).

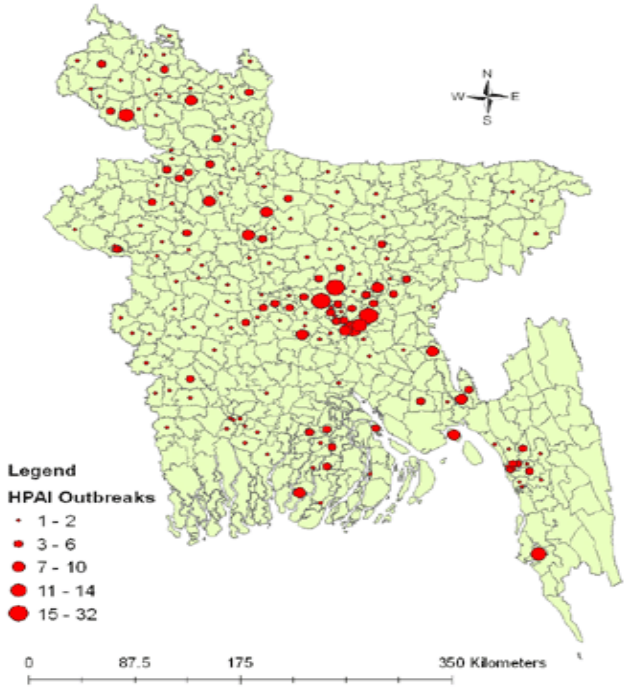


Figure 1.4. Affected districts of Bangladesh with confirmed HPAI H5N1 outbreak in poultry (OIE, 2017)

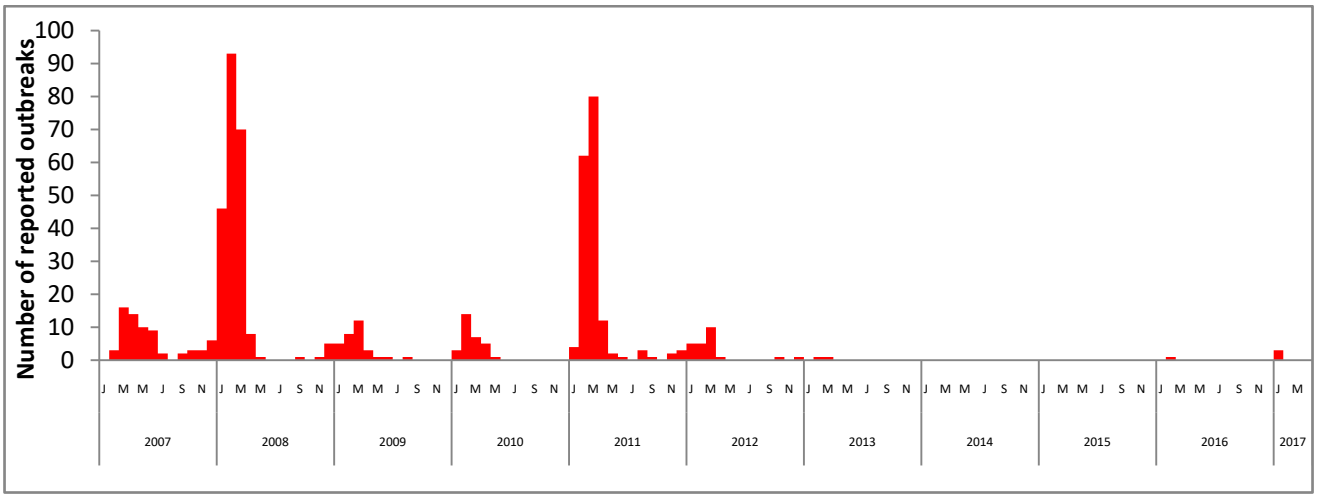


Figure 1. 5. Number of reported H5N1 outbreaks in poultry and wild birds from Bangladesh, January 2007- April 2017 (OIE, 2017)

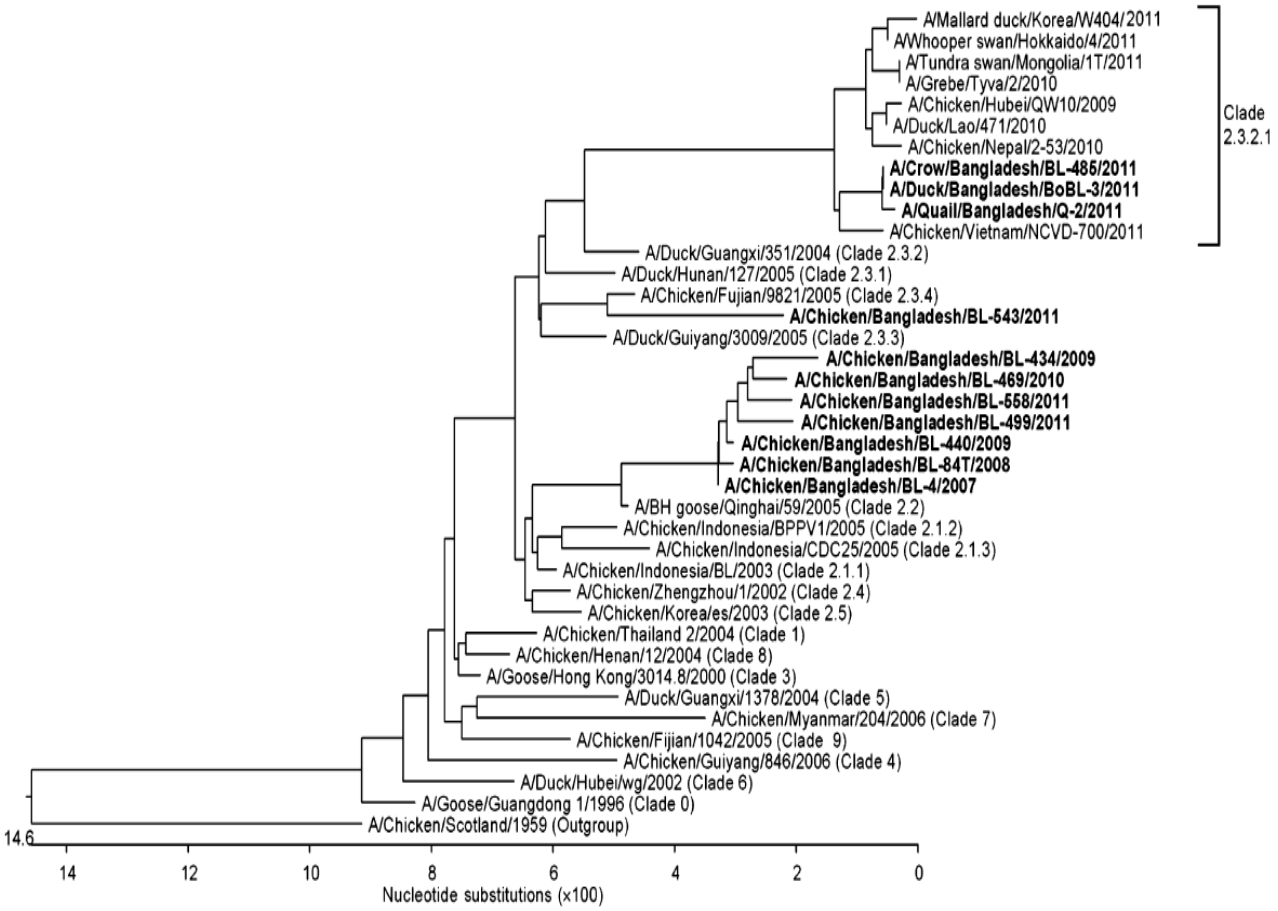


Figure 1.6. Phylogenetic tree showing different clades of H5N1 HPAI isolates from Bangladesh (Islam et al., 2012)

Avian influenza virus continues to spread in backyard and commercial poultry in many developing countries. According to the FAO, HPAI is endemic in poultry populations in Bangladesh, India, China, Indonesia, Vietnam and Egypt (El Masry et al., 2014). Despite of extensive epidemiological research conducted in Bangladesh since the first H5N1 reported outbreak in poultry, limited data are available about the genomic composition of both HPAI and LPAI viruses in Bangladesh. We need a better understanding on antigenic variation of Bangladesh isolates with others isolates detected in other countries. This study analyzes the molecular epidemiology and full genome sequences of isolated HPAI and LPAI viruses detected during 2007 to 2016 through our AI surveillance platform. The findings of this study will help public health policy makers develop and update candidate vaccine viruses for future pandemic preparedness.

1.1.7. Role of Live Bird Markets in the Epidemiology of Avian Influenza

Live Bird Markets can play an important role for the maintenance, amplification, dissemination and transmission of AIVs among avian species (Shortridge et al., 1998). Live bird markets were also identified as major source of human HPAI H5N1 influenza virus infection (Wan et al., 2011). Avian influenza subtypes H5N1, H5N2, H7N9 and H9N2have frequently been identified from samples of LBM environments in many Asian countries including Vietnam, Thailand, Egypt, Indonesia, Hong Kong, China and Cambodia (Nguyen et al., 2005; Amonsin et al., 2008; Abdelwhab et al., 2010; Henning et al., 2010; Indriani et al., 2010; Wan et al., 2011; Leung et al., 2012; Murhekar et al., 2013, Chen et al., 2016; Shi et al., 2013,). Live bird markets have been considered as important source of human infections with HPAI and most cases were associated with the exposure to live poultry during visits to LBMs (Mounts et al., 1999; Wan et al., 2011; Rivers et al., 2013; Wang et al., 2013).

Live Bird Markets of Bangladesh, particularly in urban areas, act as hubs for poultry trading and are considered as an important interface for AI transmission between humans and poultry. Urban LBMs usually run for daily trading, whereas peri-urban or rural LBM run either once or twice per week. Multiple poultry species from both backyard and commercial poultry farms are usually kept together at LBMs (ICDDRB, 2013; Biswas et al., 2015; Sayeed et al., 2017). City LBMs

have both wholesale and retail stalls. Wholesale markets usually don't slaughter poultry within their shops, however, its stalls usually remained opened for 24 hours and 30 days of a month without following any weekly or monthly rest day. Therefore, wholesale markets are at high risk and considered as a reservoir for AIVs. In contrast, retail stalls sell poultry directly to the consumers. Retail shops often slaughter poultry within their shops. Avian influenza viruses were quite frequently identified in samples collected from LBM and other poultry production sectors of Bangladesh. Avian influenza subtypes H5N1 and H9N2 were more commonly detected in LBM bird species or LBM environment. In LBM bird species, H5N1 was frequently detected in ducks and H9N2 was mostly detected in chickens and quails (Turner et al., 2017). Multiple reassortments among HPAI H5N1 viruses have been reported in avian samples collected from LBMs (Gerloff et al., 2014). Antigenic analysis revealed the predominant clade for H5N1 was 2.3.2.1 since 2012 (Gerloff et al., 2014; Marinova-Petkova et al., 2014).

Food markets were identified as an important place for HPAI H5N1 in human, poultry and environment of LBM in China. There was an evidence of H5N1 gene in wire cage for birds and neutralizing antibody against H5N1 in human (Wang et al., 2006).

Bangladesh has a large number of LBMs where multiple poultry species from backyard and commercial production systems are predominantly kept together for sale. Studies have detected AIVs in market poultry and in the environment of LBMs in Bangladesh (ICDDRB, 2013; Biswas et al., 2015). Few LBM-based epidemiological studies were conducted to identify AIVs in poultry and to assess market-level biosecurity practices in Bangladesh. But no studies were conducted to assess shop-level biosecurity practices in relation to environmental contamination with AI in Bangladesh to the best of my knowledge. One of the aims of this study was to estimate the prevalence of environmental contamination for AI and to identify the association between biosecurity practices and environmental contamination with AI.

1.1.8. Occurrence of Human Cases with Highly Pathogenic Avian Influenza

HPAI H5N1 in humans was first reported in 1997 in Hong Kong and reappeared again in 2003 in Hong Kong, China and Vietnam (Chan, 2002). Later this H5N1 spread to other parts of Asia,

Africa and Europe (Yuen et al., 1998). As of 16 May 2017, a total of 859 human cases with H5N1 infection were reported from 16 different countries between 2003 and 2017 (Appendix II: https://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). The case fatality rate was 53% (WHO, 2018). Most human cases were identified in winter and the winter peak occurred across the world. There was no significant variation between hemisphere and season in the occurrence of H5N1 outbreaks in humans (Mathur et al., 2014). Affected countries with confirmed human cases of H5N1 AI since 2003 have been presented in Figure 1.7.

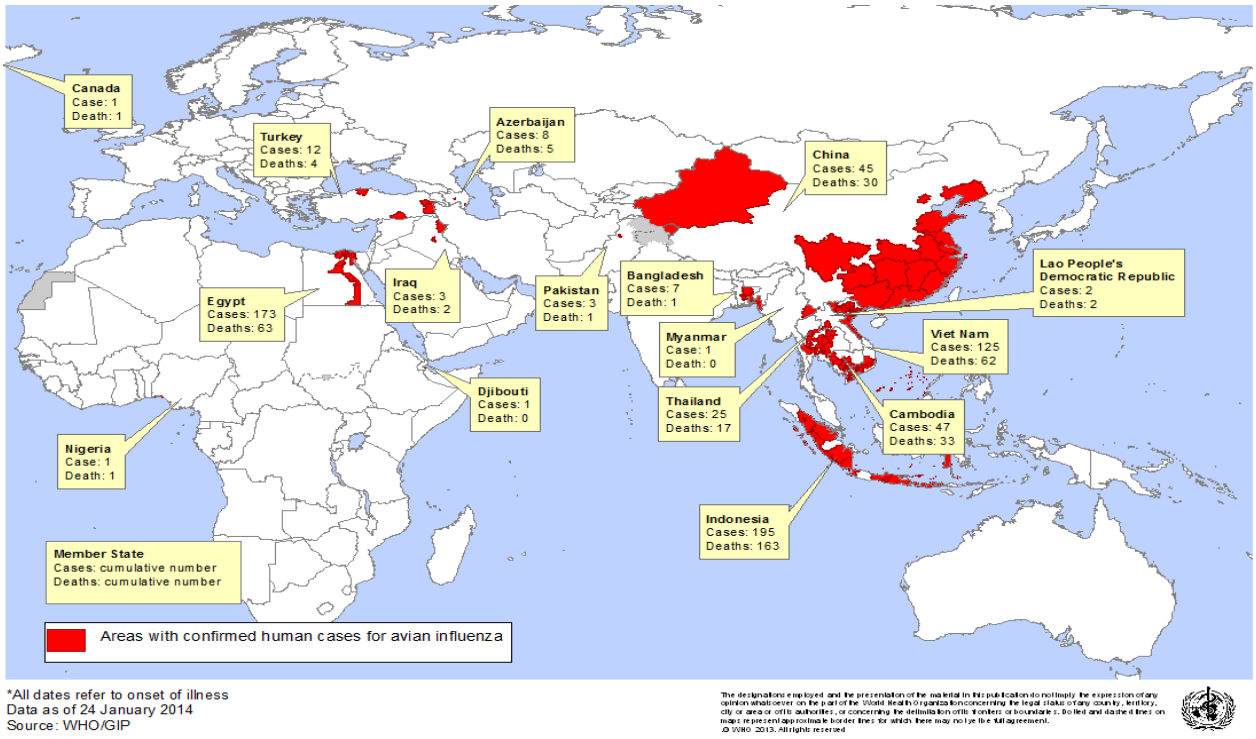


Figure 1.7. Affected countries with confirmed human cases of H5N1 avian influenza since 2003

A total of eight human cases with HPAI H5N1 infection have been reported so far in Bangladesh. Most cases were clinically mild except one death. Among the reported cases, three were poultry workers (Brooks et al., 2009; IEDCR, 2012a; IEDCR, 2012b; IEDCR, 2013). Poultry workers from several LBMs in Bangladesh were found sero-positive (2%) against H5N1 virus at baseline and 2% of poultry workers were sero-converted. Poultry workers who were involved with feeding poultry, cleaning feces from pens, cleaning food or water containers, and

not washing hands after touching sick poultry had a 7.6 times higher risk for infection compared with workers who infrequently performed these behaviors or practices (Nasreen et al., 2015).

H7N9, a novel reassorted AIV, was first identified in humans in China during February and March 2013. H7N9 virus can bind to both avian type (α 2,3-linked sialic acid) and human-type (α 2,6-linked sialic acid) receptors. The lower respiratory tract is affected by H7N9 (Zhou et al., 2013). A majority of the infected persons (82%) had a history of exposure to live animals including chickens. Few of the patients were poultry workers (Li et al., 2014). However, this subtype has fortunately not been introduced to poultry or humans in Bangladesh.

1.1.9. Avian Influenza Surveillance Systems in Bangladesh

In Bangladesh, poultry production has rapidly been growing since 1990 (Jabbar et al., 2007) and there is about USD two billion investment in this promising sector (Ali and Hossain, 2012). In the last two decades, more than 100,000 small and medium scale poultry farms have arisen in Bangladesh (GoB, 2008). In contrast, HPAI H5N1 is a serious zoonotic pathogen that poses a threat to human health and causes severe economic losses of the poultry industry in Bangladesh. Approximately 2.58 million poultry were culled due to multiple H5N1 outbreaks and the estimated financial loss was approximately USD 154 million during 2007 to 2012 (WB, 2013). In 2006, the government of Bangladesh prepared a National AI and human pandemic influenza preparedness and response plan. In Bangladesh Department of Health, Department of Livestock Services (DLS), World Health Organization, FAO and other stakeholders worked collaboratively to develop this plan. As a part of influenza preparedness and response plan, the DLS, with the collaboration of other partners and donor organizations (World Bank), strengthened the existing passive surveillance system and initiated an active surveillance programme to detect HPAI H5N1 outbreaks in both commercial and backyard poultry. The main objective of this surveillance is to identify outbreaks rapidly and to prevent the transmission of HPAI.

FAO conducted active surveillance for HPAI H5N1 in 150 out of 487 sub-districts in Bangladesh with the funding support from USAID in 2008. A total of 450 Community Animal Health Workers (CAHW), 50 Additional Veterinary Surgeons (AVS) and 150 Upazilla Livestock

Officers (ULOs) were trained to conduct this surveillance. A Short Message Service (SMS) gate way (i.e. method of sending and receiving SMS messages between computers and mobile phones) was introduced to collect data and report on morbidity and mortality in poultry. At the end of the day of farm visits, each CAHW sends a SMS message with the total number of all investigated poultry (chickens, ducks and other birds) and their health status (the number of sick and dead birds) to the SMS gateway system. A central surveillance team at the DLS reviewed the internet based SMS outputs to monitor trends in disease, morbidity and mortality in poultry. This real-time reporting using SMS was found to be effective in identifying HPAI H5N1 outbreak rapidly to then inform response and control efforts (FAOAIDE, 2009).

In line with government surveillance, International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) has also been performing a LBM based sentinel surveillance for AI in poultry in 2007. icddr,b signed a Memorandum of Understanding with the DLS, Bangladesh to collaborate on specimen and data collection, diagnosis, training, and research on AI. The primary objective of LBM based AI surveillance is to identify AI strains that are circulating in domestic poultry within Bangladesh. Centers for Disease Control and Prevention, USA has been providing funding and technical support from the beginning of this surveillance. Department of Livestock Services, Bangladesh is the key stakeholder of this surveillance. Initially LBM in the Mohonganj upazila of Netrokona district was selected for sampling and data collection from poultry. Then the surveillance was expanded to other sites, including Dhaka, Gazipur, Rajshahi, Dinajpur and Chittagong. The primary goal of the surveillance was to detect HPAI H5N1 from poultry in LBMs and backyard farming in the rural and peri-urban areas. Passive surveillance also went on with the aim of collecting specimens from poultry suspected with the HPAI and to analyze the specimens for characterization of strains that are currently circulating. With the consistent funding support from USA CDC, the surveillance programme is still going on (ICDDRB, 2011).

In 2016, the animal and human health services of the Government of Bangladesh in collaboration with FAO developed a novel method to detect AIVs using pooled environmental samples in LBMs in the pathogen sink area of Dhaka and then expanded to other cities in Bangladesh. A joint team of animal health and human health government officers visited 106 LBMs on a

monthly basis to collect environmental pool specimens. This surveillance system is still ongoing (Brum et al., 2016).

In 2012, icddr,b, in collaboration with Institute of Epidemiology, Disease Control and Research (IEDCR), commenced poultry worker surveillance to identify AIVs among poultry workers who were working in LBMs. The objectives of this surveillance were (i) to identify LBM workers and their household members who were infected with AIVs, (ii) to measure the incidence of sero conversion of AI virus antibodies among LBM poultry workers and their household members, (iii) to measure the incidence of laboratory confirmed AI infection among the LBM poultry workers and their household members, (iv) to characterize the AI virus strains causing laboratory confirmed infections among the ill poultry workers or their household members and to compare these strains to those currently circulating among poultry in LBMs and (v) to validate a novel H5 rapid serology diagnostic test to detect H5 antibodies among people with confirmed exposure to H5N1 virus (Afreen et al., 2013).

1.1.10. National Avian Influenza Preparedness and Response Plan

The Government of Bangladesh has developed the National AI Preparedness and Response Plan with technical support from WHO, FAO and UNICEF. The main goal of this plan is to prevent and control avian and pandemic influenza and to prepare for reducing morbidity and mortality in both poultry and humans with the aim of minimizing socio-economic and environmental impact. Five strategies have been pointed out to achieve the objectives of this plan. These strategies include planning and coordination, surveillance, prevention and control, risk communication, and operational research. Multiple committees were formed to implement the plan and their details are given in Appendix III.

1.1.11. Effects of Climatic Factors on the Incidence of Avian Influenza

Global climate change can influence bird migration, the AIV transmission cycle and virus survival outside the host. However, very little is known about the direct effect of environmental factors on AI transmission and persistence. Avian influenza outbreaks and high prevalence of AI

were observed in winter, because the higher virus persistence in cold water may promote AIVs transmission (Gilbert et al., 2008).

The risk factors for the 2006-2007 outbreaks in Vietnam were due to the keeping medium level poultry density and rainy seasons. The study suggested extensive rain resulted temporal flooding that could promote re-emergence of HPAI (Henning and Pfeiffer, 2009).

In Europe, the occurrence of HPAI H5N1 in wild birds was highly correlated with the increased normalized difference vegetation index (NDVI) in December; intermediate NDVI in March; lower elevations; increased minimum temperatures in January; and reduced precipitation in January (Si et al., 2010).

A case-control study from China found that the occurrence of 128 HPAI H5N1 outbreaks in poultry and wild birds as well as 21 human cases was significantly associated with minimal distance to the nearest national highway, annual precipitation and the interaction between minimal distance to the nearest lake and wetland. The risk of HPAI H5N1 outbreaks was increased when the precipitation in a region was decreased (Fang et al., 2008).

Bangladesh is sub-tropical country which has three main seasons in a year which are summer (March-May, average minimum temperature 22.4°C to maximum 32.6°C), monsoon (June-September, average minimum temperature 25.5 °C to maximum 31.5°C) and winter (December-February, average minimum temperature 13.9 °C to maximum 26.5°C). The average annual rainfall varies from 1,429 to 4,338 millimeters across different seasons (BBS, 2015b).

Spatio-temporal pattern of AI was previously studied using AI outbreak data in different poultry sectors in Bangladesh (Ahmed et al., 2011). The peaks of the HPAI H5N1 outbreak waves were reported to occur in February-July in 2007 and January-April in 2008 in Bangladesh (Ahmed et al., 2010). However, another study found no correlation between climatic factors and HPAI H5N1 outbreaks in poultry (Biswas et al., 2014). As most of the AI outbreaks were reported during winter months (December-February), the analysis for temporal pattern of AI was not clearly understood for a year. These findings warrant further study to identify the actual

relationship between climatic factors and the occurrence of AI. Year-round surveillance data is therefore more informative and powerful than outbreak data to understand a clear temporal pattern of AI circulation in Bangladesh. Also, the association between AI circulation and climatic parameters was not previously analyzed to assess the relationship in Bangladesh. Therefore, it was aimed to conduct a study to investigate AI at time and space incorporating climatic parameters in different poultry species (waterfowl, commercial and backyard chicken) using a big data set produced through the sentinel surveillance programme in 2007-2016 with the aim of developing intervention strategies for preventing and controlling AI in poultry and humans in Bangladesh.

1.1.12. Economic and Public Health Consequence

Highly Pathogenic Avian Influenza infection causes serious economic loss throughout the world. The losses are due to the depopulation of infected and exposed poultry, unemployment in the poultry industry, costs of eradication, quarantine restrictions, reduction of meat and egg prices, costs for improving biosecurity practices and costs for vaccination in poultry. The poultry industry is growing rapidly, particularly in developing countries. A lot of manpower is involved in this industry. The economic consequences are greater in Asian countries where AI is endemic. In 2004, approximately 12% of all domestic birds were either died or culled to prevent the spread of disease in Vietnam. In 2005, WHO estimated that 35% of world's population can be infected due to the spreading of flu throughout the world in as few as 180 days. A predictive model suggested that the total cost to the world would be more than $2.0 trillion if a moderately severe pandemic happens. In the case of severe pandemic, the total cost would be $3.13 trillion (Burns et al., 2006). Two AI outbreaks in USA have caused economic loss around US$ 65 million for disease control and US$ 140 million due to the loss of poultry (FAO, 2004b).

In Bangladesh, more than 100,000 of small and medium scale poultry farms were established across the country in the last two decades. The impact due to the HPAI outbreaks during 2007 to 2008 was assessed. Over 1.6 million birds were destroyed from 547 commercial and 42 backyard flocks. Layer flocks (78%) were more often infected than broiler flocks (22%). The price of a

day-old chick (DOC) fell from US$0.50 to 0.07. Estimated losses were around US$9.88 million or Taka 687 million (Chakma and Rushton, 2008).

1.1.13. Diagnosis of Avian Influenza

Virus isolation is the gold standard for AI detection. HPAI viruses can be isolated in embryonated eggs or in cell culture, using permissive cells such as Madin Darby Canine Kidney (MDCK) cells or rhesus monkey kidney (LLC-MK2) cells. Cytopathic effects in cell culture are non-specific in nature. Biosafety level 3 laboratory facilities or higher are required for virus isolation (de Jong and Hien, 2006).

Avian influenza can be screened by immunofluorescent staining with monoclonal antibodies against the nucleoprotein. Subtype-specific reverse transcription polymerase chain reaction (RT-PCR), hemagglutination inhibition (HI) and neuraminidase inhibition assays are used for further subtyping of HA and NA (de Jong and Hien, 2006). Swabs from throat, nasal secretions or washings and conjunctival swabs are used for isolation of AIVs in humans (Fouchier et al., 2004; Hien et al., 2004). Virus was also isolated from serum, cerebrospinal fluid, and rectal swabs (de Jong et al., 2005).

Real time reverse transcriptase PCR assay is used for specific detection of viral nucleic acids. RT-PCR is reliable in terms of high sensitivity (95.6%) and specificity (96.3%) and test results can be generated within a few hours after sample collection(Cattoli et al., 2004). Real time RT-PCR is considered as the best method for outbreak of AI (Chotpitayasunondh et al., 2005).

Hemagglutination inhibition assays are the gold standard for detection of antibodies against influenza viruses (de Jong and Hien, 2006). However, the HI assay failed to detect antibodies against AIVs in mammals, even in cases where infection was confirmed by virus isolation. Studies suggested this failure of detection could be due to the poor immunogenicity of some AIVs and lack of sensitivity to detect low titer or less antibodies induced by viruses (Hinshaw et al., 1981; Kida et al., 1994; Lu et al 1982).

1.1.14. Prevention and Control

According to the OIE guideline, the control strategy for HPAI in poultry is a combination of several measures; stamping out, movement restrictions and emergency vaccination. Stamping out is a control measure that includes culling infected poultry and non-infected poultry that have had contact with infected premises or are located close to an infected premises. Environmental contamination can be reduced significantly by movement restrictions of infected birds, improved hygiene and biosecurity of farms, and appropriate surveillance (OIE, 2004). Culling both infected and exposed healthy poultry were found to be the most effective control measures during AI outbreaks in Hong Kong, the Netherlands and Canada (Chan, 2002; Tweed et al., 2004).

In Bangladesh, the government implemented a weekly rest day practice for cleaning and disinfection of Dhaka city LBM environment in April 2012 to reduce environmental contamination (Biswas et al., 2015). Moreover, the Government of Bangladesh (DLS) and Breeder Association of Bangladesh introduced AI trial vaccine for the first time in commercial poultry farms at the end 2012. This vaccine was targeted for layers, broilers and breeders and applied at day-old chicks at hatcheries. The cost for a single dose of the vaccine was approximately 5 taka (GoB, 2013).

Controlling AI in poultry is crucial to prevent infection in human. Many risk factors have been identified for H5N1 infections in poultry. Duck and geese carry H5N1 virus without showing illnesses. They amplify viruses and spread viruses to the other susceptible poultry species (Henning et al., 2010). So, chicken should not be reared with duck and geese. In Hong Kong, live ducks, geese and quails were banned in retail LBMs to control the spread of H5N1(Guan et al., 2007).

A vaccination programme has also been recommended to control HPAI. An experimental study showed that vaccination protected against clinical signs, reduced mortality, lowered virus shedding and increased resistance to infection in birds (Capua et al., 2004; EC, 2003). However,

the virus can replicate and cause illnesses in vaccinated birds. These findings indicated that vaccination alone has not been successful to prevent or control or eradicate AI in birds.

The different infrastructures of the poultry industries and LBMs in some Asian countries made a vaccination campaign infeasible. That's why HPAI is endemic in vaccinated poultry populations in some Asian countries. To prevent AI outbreaks in endemic countries, biosecurity practices need to be upgraded along with implementation of vaccination campaigns. If the vaccination programme is not properly managed with upgraded biosecurity, prevent or control or eradication of AI will not be possible and there is a possibility of a public health threat in the future (Capua and Marangon, 2004).

1.1.15. Conclusion

The above discussion has identified a knowledge gap on patterns of AIVs circulation in domestic poultry, tempero-spatial distribution of AIVs with climatic association and biosecurity practices in regards to environmental contamination with AI at LBMs.

Avian influenza surveillance is useful and effective to identify AIVs including novel subtypes in domestic poultry. The role of LBM on the epidemiology of AI is critical. LBM is considered as an important place for animal-human interface in the emergence of AIVs. Live Bird Market-based surveillance is demonstrated as a low-cost and simple platform. Thus, LBM-based active surveillance is an important platform to understand the pattern of AIVs, to assess the molecular changes of AIVs over time and for early detection of HPAI including novel influenza strains of public health importance.

Until today, very limited data were published on the role of meteorological factors in the occurrence of circulating AIVs in avian species. Information about meteorological factors in relation to AI circulation is necessary for successful planning of control and prevention strategies in high-risk areas considering AI seasonality.

The role of biosecurity practices including cleaning and disinfection is important to reduce environmental contamination as well as to prevent AIV transmission at LBM. An epidemiological study is needed to identify certain risky biosecurity practices that can be targeted for improvement to reduce AI transmission and environmental contamination in LBM settings.

The present PhD research has been conducted to study the dynamic patterns of AI circulation in poultry sold at LBMs and factors associated with the risk of AI circulation in LBM.

1.2. Thesis Structure

The thesis has five individual chapters. We prepared chapters 2-4 with the aim of publishing study findings in scientific journals. **Chapter 1** provided a general introduction on AI with relevant information instead of two separate chapters as "**Introduction**" and "**Literature Review**" with the consultation of PhD supervisory team. This thesis has not included any independent chapter named "General Methodology", specific materials and methods are described in each main chapter (**Chapters 2, 3 and 4**). We have followed the reference style described in CVASU thesis guidelines in the thesis. A brief summary of each chapter is organized as follows.

1.2.1. Chapter 1: General Introduction

This chapter provides detailed information of existing published and un-published articles and information on AI epidemiology. This literature review helped identify the scientific gap that influenced me to conduct this study on dynamic patterns of AI circulation in poultry sold at LBMs and factors associated with the risk of AI circulation.

1.2.2. Chapter 2: Monitoring Avian Influenza Viruses in Domestic Poultry through a Sentinel Surveillance at Live Bird Market in Bangladesh

Active surveillance is useful to detect AIVs circulation in poultry. Findings of the surveillance are important for local preparedness and control activities. This chapter provides a better understanding on ongoing surveillance system and regular activities. This LBM-based AI surveillance provided detailed information on the pattern of AIV circulation in poultry populations. It also improved knowledge on molecular analysis of AIVs detected in the surveillance platform.

1.2.3. Chapter 3: Avian Influenza Viruses Seasonality in Domestic Poultry and its Association with Climatic Factors in Bangladesh

In spite of extensive research works on AI epidemiology, the seasonal drivers of AI occurrence in domestic poultry in Bangladesh are not clearly understood. Year-round surveillance data are more powerful than any seasonal outbreak data to understand the temporal pattern of AI circulation. In this study, we used ten years of surveillance data to investigate AI at time and space incorporating climatic parameters in different poultry species (waterfowl, commercial and backyard chicken). Findings of this study are important to develop intervention strategies for preventing and controlling AI in poultry and humans in Bangladesh.

1.2.4. Chapter 4: Association between Biosecurity Practices and Environmental Contamination with Avian Influenza Viruses in Live Bird Markets, Bangladesh

This chapter provides information on the whole biosecurity system of poultry shops in regards to environmental contamination with AI. Findings of this study are useful to identify certain biosecurity practices for reducing environmental contamination within LBM.

1.2.5. Chapter 5: General Discussion

This chapter provides overall discussion on key research findings from the whole body of research works (**Chapters 2-4**) and its relevance to the global scientific research findings. The discussion was constructed based on the results that tempted to draw all possible interpretations and potential implications. This chapter clearly mentions some study limitations with its effect on study findings. This chapter also provides specific recommendations and future direction with the aim of controlling and preventing AI in Bangladesh.

1.2.5. Appendix in brief

Appendices include supplementary tables for different chapters, list of published abstracts in different conferences and a list of seminar presentations.

1.3. Aims of the Research Project

1.3.1. Specific Objectives of the Project

1) To monitor avian influenza viruses and their statuses in domestic poultry through Live Bird Market-based sentinel surveillance in Bangladesh
2) To determine avian influenza viruses seasonality in domestic poultry and its association with climatic factors in Bangladesh
3) To estimate the association between biosecurity practices and environmental contamination with avian influenza viruses in Live Bird Markets in Bangladesh

1.3.2. Outcomes of this Project

1) Detected avian influenza viruses that were circulating in domestic poultry and understood the spatio-temporal distribution of avian influenza viruses
2) Identified climate factors associated with the circulation of avian influenza viruses in domestic poultry
3) Identified certain risky modifiable biosecurity practices of LBM to target for reducing environmental contamination of live bird market

Chapter-2: Monitoring Avian Influenza Viruses in Domestic Poultry through a Sentinel Surveillance at Live Bird Market in Bangladesh

2.1. Introduction

Highly Pathogenic Avian Influenza (HPAI) A virus (H5N1) poses a threat to the different poultry sectors, wildlife health and human health (OIE, 2018; WHO, 2018). Over the last two decades, H5N1 has caused a large number of outbreaks in poultry in Asia, Europe and Africa (OIE, 2018: http://www.oie.int/en/animal-health-in-the-world/update-on-avian-influenza/2016/). Chickens are susceptible to HPAI H5N1 with high morbidity and a case fatality rate as high as 100% (Alexander, 2007). Asymptomatically infected ducks or aquatic birds could serve as reservoirs for sustaining and perpetuating H5N1 and other avian influenza viruses, and remain undetected while maintaining the ability to transmit infection (infectiousness) to other susceptible hosts (Hulse-Post et al., 2005; Kim et al., 2009b). Avian influenza viruses can be transmitted from bird to bird through direct contact, and indirectly by exposure to contaminated fecal material, aerosols, water, feed and other materials (de Jong and Hien, 2006). As of March 2018, a total 860 cases of H5N1 in humans have been reported, with a case fatality rate of >53% (WHO,2018:https://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/). Many epidemiological studies have found that most human cases of H5N1 infection had a history of poultry exposure including slaughtering and consumption of sick poultry and handling of infected live and dead poultry (Mounts et al., 1999; Chan, 2002; Beigel et al., 2005; Dinh et al., 2006).

Bangladesh is the 8th most populous country in the world, with one of the highest population densities of nearly 976 persons per square kilometer (as of 2011) (BBS, 2015a). Domestic poultry are raised throughout Bangladesh and 90% of rural households raised poultry (Sonaiya et al., 2004). The first HPAI outbreak was reported in Bangladesh in March 2007 and there have been more than 550 reported HPAI H5N1 outbreaks to date, 90% of which were in commercial poultry farms (OIE, 2018). So far, this virus has spread in many parts of the country. HPAI H5N1 outbreaks were detected in 52 out of 64 districts(OIE, 2018). This virus continues to be

identified in wider regions and introduction of the virus by wild migratory aquatic birds cannot be completely ruled out as the country is situated on a major flyway of migratory wild birds (East Asian-Australasian Flyway and Central Asian Flyway). It may suggest that the virus is already wide-spread in many parts of the country but yet to be detected. The people of Bangladesh live in close proximity with their poultry. This coexistence is continuous and close, enabling recurrent encounters of people with AIVs from poultry or animals (GoB, 2011). So far, eight human cases, including one death, have been reported in Bangladesh (Brooks et al., 2009; IEDCR, 2012a; IEDCR, 2012b; IEDCR, 2013).

Early detection of AIVs in poultry, followed by rapid and safe culling of infected and exposed poultry and characterization of the virus strains could avert an influenza pandemic. In Bangladesh, AI surveillance is necessary for local preparedness and control activities. First, it will detect avian influenza viruses among backyard and commercial poultry. Second, it will identify the unusual poultry die-offs due to HPAI H5N1 in Bangladesh. Third, it will provide material that can be used to determine the virus' phylogenetic relationship to the dominant genotypes that are circulating elsewhere in Asia, providing additional clues as to how the virus is spreading and evolving. Fourth, it will provide materials that can be used to help develop appropriate vaccines that address the growing genetic diversity of the H5N1 genotypes, which currently make single vaccine solutions an untenable global prevention strategy. Overall, these findings will help public health officials understand the importance of ongoing avian influenza surveillance among the poultry population in Bangladesh.

Live bird markets (LBMs) are a potential source for HPAI H5N1 in many Asian countries including Vietnam, Thailand, Indonesia, Hong Kong, China and Cambodia (Nguyen et al., 2005; Amonsin et al., 2008; Abdelwhab et al., 2010; Indriani et al., 2010; Wan et al., 2011; Leung et al., 2012; Horm et al., 2013). In Bangladesh, multiple poultry species including domestic chickens, ducks, geese, pigeon, and quail are reared together in backyard farms and sold in LBMs, which facilitates transmission between species. Urban markets trade poultry every day, whereas semi-urban rural markets have retail and wholesale poultry stalls trade poultry twice weekly. Understanding the circulation of different strains of AI in the poultry population would provide insight on the transmission process of this virus. We therefore aimed to carry out a

LBM-based surveillance programme to estimate the level of AIVs and their distribution, as well as associated risk factors and phylogenetic characteristics in domestic poultry including waterfowl, commercial chicken and backyard chicken.

2.2. Materials and Methods

2.2.1. Study Sites and Study design

The Zoonotic Diseases Research Group of International Centre for Diarrhoeal Disease Research, Bangladesh (icddr,b) commenced LBM based active AI surveillance in Bangladesh in October 2007 when the outbreak of HPAI H5N1 occurred in different poultry production sectors in this country. With the appropriate permission (MOU between icddr,b and CVASU, Date of approval: 6 November 2013), I have used 10 years data from this surveillance platform for my PhD thesis. This AI surveillance at LBMs initially started with Chittagong, Rajshahi and Netrokona and was then extended to Dhaka, Gazipur, Kishoreganj, Jessore, Faridpur, Dinajpur and Bogra (Figure 2.1). The study sites were chosen based on the previous occurrence of HPAI H5N1 outbreaks, highest volume of multiple poultry species trading or transaction and size of the market as well as AI vaccination coverage (Herald, 2013; OIE, 2017).

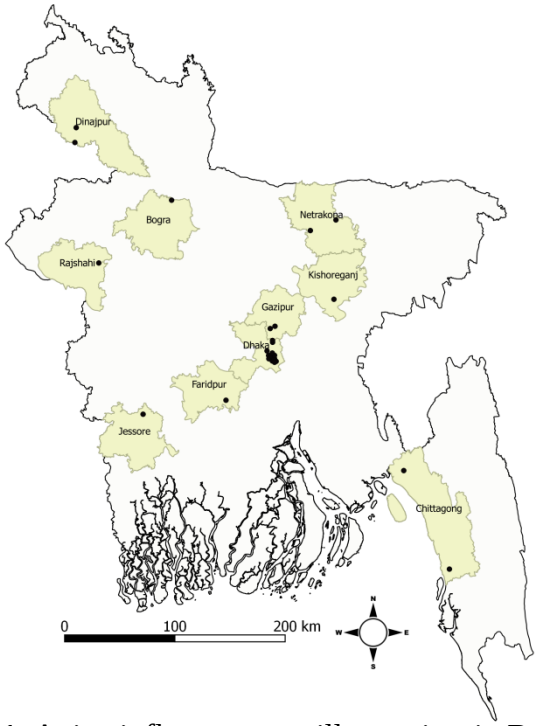


Figure 2.1. Avian influenza surveillance sites in Bangladesh

2.2.2. Selection of Live Bird Markets, Sample Size and Sampling Period

One peri-urban large LBM from each of four selected districts (Chittagong, Netrokona, Rajshahi and Dinajpur) was selected for waterfowl sampling (Table 2.1). Peri-urban LBMs were selected because of the availability of sufficient domestic waterfowl in the market. The surveillance team collected samples from 20 waterfowl from each LBM monthly.

A total of 24 LBMs (16 from Dhaka city, 3 from Gazipur city, 1 from Kishoreganj, 1 from Chittagong, 1 from Netrokona, 1 from Rajshahi and 1 from Dinajpur) were selected for commercial chicken sampling (Table 2.1). We selected these LBMs for commercial chicken sampling because these LBMs are considered as hubs that receive commercial chicken from many commercial farms located in different parts of the country. The surveillance team collected samples from 5-15 commercial chicken from each LBM during monthly visits.

Five peri-urban LBMs from five districts (Chittagong, Netrokona, Jessore, Faridpur and Bogra) were considered for backyard chicken sampling (Table 2.1). These peri-urban LBMs were selected because of the availability of sufficient number of backyard chicken in the market. The surveillance team collected samples from 10 backyard chickens from each LBM by monthly visit.

For environmental sampling, a total of 29 LBMs (16 Dhaka city, 3 Gazipur city, 2 Chittagong, 2 Netrokona, 1 Kishoreganj, 1 Rajshahi, 1 Dinajpur, 1 Jessore, 1 Faridpur and 1 Bogra) were selected from 29 peri-urban and urban sites (Table 2.1). The surveillance team collected one environmental pool sample from each LBM by monthly visit.

Table 2.1. Distribution of live bird markets by district and distribution of samples by markets, types of samples and sampling period

Districts	No of urban LBMs	No of peri-urban LBMs	Bird species	Environmental sample	Sampling period
Dhaka	16	-	Commercial chicken	Yes	2009-2016
Chittagong	-	2	Waterfowl, commercial and backyard chicken	Yes	2007-2016
Kishoreganj	1	-	Commercial chicken	Yes	2013-2016
Netrokona	-	2	Waterfowl, commercial and backyard chicken	Yes	2007-2016
Jessore	-	1	Backyard chicken	Yes	2013-2016
Faridpur	-	1	Backyard chicken	Yes	2013-2016
Rajshahi	-	1	Waterfowl and commercial chicken	Yes	2007-2016
Gazipur	3	-	Commercial chicken	Yes	2013-2016
Dinajpur	-	1	Waterfowl and commercial chicken	Yes	2009-2016
Bogra	-	1	Backyard chicken	Yes	2013-2016
Total	**20**	**9**			

2.2.3. Operation of Active Surveillance

2.2.3.1. Market Visit and Sample Collection, Preservation and Transportation

A field team consisting of a registered veterinarian and a field assistant visited the markets once every month on a market day. Waterfowl markets usually sit twice in a week. The field team informed the waterfowl owners about the surveillance objectives and sampling procedure. The field team enrolled waterfowl from those owners who agreed to provide written consent for collecting samples from their waterfowl. Accordingly, the team sampled 20 waterfowl per market in a visit using non probability sampling technique. A maximum of four waterfowl were enrolled from an individual owner or vendor depending on the availability of waterfowl. The

team collected one of the following three types of samples from each selected waterfowl: cloacal swab, swabs from freshly laid (or voided) feces, or oropharyngeal swabs. When the field team found any waterfowl showing signs of respiratory illness, such as ocular or nasal discharge or swollen infra-orbital sinuses, they collected an oropharyngeal swab only. All samples collected from individual waterfowl were stored in viral transport media (VTM).

Commercial chickens were sampled from 24 LBMs. The field team visited each LBM to collect cloacal swabs from 5-15 commercial chicken once in a month using non-probability sampling technique. Commercial chickens included broilers, layers and breeders (spent parent stock). During chicken selection, dead or sick chicken were given priority for sampling. If we did not find any sick or dead chicken, we selected apparently healthy chicken. All samples collected from individual commercial chickens were stored in VTM.

Backyard chickens were sampled from five backyard chicken markets (Chittagong, Netrokona, Jessore, Bogra and Faridpur). The field team visited each market once in a month to collect specimens from backyard chicken. The team sampled 10 backyard chickens per market conveniently in a market day. Maximum three backyard chickens were enrolled from individual owners, depending on the availability of chickens in the markets. All samples collected from individual backyard poultry were stored in VTM.

Environmental samples were obtained from all urban and peri-urban LBMs. One pooled environmental sample from each of LBMs was collected once in a month. Each pooled environmental sample was prepared by swabbing seven surfaces including poultry droppings, cages, feed, water, slaughtering sites, market floors and drains. We used 8-10 swab sticks to collect specimens from seven surfaces. These 8-10 swabs from a market were mixed within a vial containing 50 ml VTM to prepare a pool.

All individual samples and pooled environmental samples were placed in a cold box and maintained at 2-4°C for up to 72 hours at field sites before being transported to icddr,b where they were immediately transferred to a -80° C laboratory freezer.

2.2.3.2. Data Collection

Each poultry raiser or vendor of sampled poultry was interviewed using a structured questionnaire about poultry demographics, poultry husbandry, flock size, health status, and any flock mortality during the past seven days and address of the poultry owner/vendors. The detailed questionnaire is given in Appendix-IV, V and VI. In most cases, we enrolled poultry from poultry raisers. Due to insufficient number of poultry during the market day, we enrolled poultry from vendors in some cases to reach the target required sample size. Poultry vendors from peri-urban markets usually buy poultry from local market and sell to the urban markets. They keep poultry in their households for certain period and wait for selling. In those circumstances, we collected husbandry, mortality and other data of poultry that they keep in their households for a short period of time.

2.2.3.3. Laboratory Evaluation

Molecular evaluation of AI was performed at the icddr,b virology laboratory. Total RNA was recovered from 100 µl of swab specimen collected in 2 ml viral transport medium (VTM) using a commercial RNA extraction kit (RNeasy Mini kit, Qiagen, Valencia, CA, USA). The RNA extract was then screened for the presence of influenza A viruses by one step reverse transcription real time polymerase chain reaction (rRT-PCR) targeting matrix (M) gene followed by the sub-type specific rRT-PCR (H5, H7 and H9) on the samples positive to the screening test using the protocols recommended by Centers for Disease Control and Prevention (CDC) (CDC, 2013; Kis et al., 2013). rRT-PCR was performed using AgPath-IDTM One-Step RT-PCR Kit (Applied Biosystems, Foster City, CA) and BioRad CFX-96 real time PCR machine. Any sample having a threshold value of 37 or less in rRT-PCR testing was considered positive. For validation of the results a subset of all influenza type A positive samples was sent to US CDC for further characterization. The details of master mixes of rRT- PCR tests, reaction conditions and the primers and probes sequences are given in Appendix VII.

2.2.3.4. Virus Isolation, Subtype Detection and Full Genome Sequencing

At the US CDC lab, samples with influenza A matrix gene ct values less than 30 were inoculated into 10-11 day old embryonated chicken eggs and amniotic or allantoic fluid was harvested 24 hours post-inoculation prior to testing by hemagglutination with turkey red blood cells to detect the presence of AI virus according to the protocol as described (Gerloff et al., 2014; Gerloff et al., 2016). All infectious materials were handled and maintained in bio-safety level-3 containment. Specimens that yielded 8 or more HA units were included for further analyses. Genomic RNA extracted from virus infected amniotic or allantoic fluid using the RNeasy extraction kit (Qiagen,Valencia,CA) was used as template for generation of cDNA by random hexamer-primed reverse transcription (SuperScript^{R}III, Life Technologies, Carlsbad, CA). The surface and internal protein genes were then amplified using influenza A virus specific primers as overlapping fragments with the Access Quickone-step RT-PCR kit (Promega, Madison, WI) and subsequently sequenced on an automated Applied Biosystems 3730 system using cycle sequencing dye terminator chemistry (Life Technologies, Carlsbad, CA). Contigs of full length open reading frames were generated for each gene (Sequencher4.10.1, GeneCodes, AnnArbor, MI). For full genome phylogenetic comparison, publicly available similar and dissimilar AI subtypes sequences in GeneBank were included in data sets and annotated according to their HA clade designation as described (Gerloff et al., 2014; Gerloff et al., 2016).

2.2.3.5. Data Analysis

The surveillance data (field and lab) were entered in SPSS-17. Data integrity was checked in SPSS before exporting to STATA-13 for epidemiological analysis. Descriptive analysis was performed on the sampled poultry population. Prevalence of influenza A viruses including their subtypes were estimated for waterfowl, commercial chicken, backyard chicken and environmental specimens. A chi-square test was applied to assess the association between frequencies of the identifying influenza A virus in sampled poultry and categories of different variables related to demography and dichotomous clinical, demographic and husbandry variables. Logistic regression analyses were performed as described by Yan Dohoo (Dohoo et al., 2003) to identify associations between different potential exposures and harboring influenza A viruses and influenza A/H5 virus. A prevalence ratio (PR) with 95% confidence intervals (CI)

was estimated to identify the significant association. Initially we performed univariable analysis to calculate PR for crude association. For further analysis, we considered only those exposure variables having p-vale <0.2 in univariable analysis to construct the final model of multivariable logistic regression analysis. We used a backward stepwise variable selection procedure to construct final model with a significance level of $p \leq 0.05$. Confounding variables were checked by re-adding, one by one variable. We considered a variable as confounder if its removal from the model made the regression coefficients of the remaining variables showed a relative change (≥15%). We also checked for possible interaction and collinearity between variables during model building. We constructed four separate models; model-1 for waterfowl, model-2 for commercial chicken, model-3 for backyard chicken and model-4 for environmental specimens. Descriptive results were expressed as frequency number, percentage, mean, median, prevalence ratio, 95% confidence interval and p value.

2.2.3.6. Ethical Considerations

The field team obtained written consent from poultry owners or poultry vendors before collecting sample from their poultry. The field team described the purpose of this surveillance, expected outcome, process of sampling, and potential harm and benefits of being included in the study. The research review committee, ethics review committee and animal experimentation committee of icddr,b reviewed and approved the surveillance protocol. The ethics approval number was 2007-10.

2.3. Results

2.3.1. Status of Avian Influenza Viruses Circulation in Waterfowl and Associated Factors

Over the surveillance period (2007-2016), a total of 7,997 waterfowls were sampled, of which ducks constituted 7,043 and geese 954. Of the sampled waterfowls, 89% were adult and 94% were apparently healthy. Most of the sampled waterfowl were raised by backyard farming (94%). The mean and median size of backyard poultry flock was 19 and 14, respectively (range: 1-100). The poultry mortality history of the past seven days of sample collection was less than 1% over the surveillance period.

Waterfowl had 6% detectable RNA for influenza A virus of which 3% were H5 subtype specific RNA. Influenza A/H5 was more frequently detected in the year 2011 and 2012 than other years. The difference for year wise avian influenza detection was statistically significant ($p < 0.001$). Among the waterfowl, ducks were three more likely to be positive for AI A viruses compared to geese (PR 3.4, 95% CI: 2.2-5.4). The domestic waterfowl which were sampled during winter season were more likely to test positive for influenza A viruses compared to the waterfowl sampled during summer and monsoon. There was no significance difference for AI detection in terms of husbandry practices. In the final multivariable analysis model, types of waterfowl and seasonality were significantly associated with avian influenza (Table 2.3).

Table 2.2. Demographics and detection of influenza virus type A and H5 subtype among waterfowl from live bird markets, Bangladesh, August 2007-December 2016

Characteristics	Total number of samples n (%)	rRT-PCR positive sample number	
		Influenza virus A (all subtypes) n (%; 95% CI)	H5 Subtype n (%; 95% CI)
Waterfowl type			
Ducks	7,043 (88)	478 (7; 6.2-7.3)	199 (3; 2.4-3.2)
Geese	954 (12)	19 (2; 1.2-3)	13 (1; 0.7-2.3)
Age of waterfowl			
Adult	7093 (89)	449 (6; 5.7-6.9)	191 (3; 2.3-3)
Juvenile	904 (11)	48 (5; 3.9-6.9)	21 (2; 1.4-3.5)
Farm type			
Backyard waterfowl (flock size: 1-100)	7546 (94)	466 (6; 5.6-6.7)	211 (3; 2.4-3.1)
Small scale waterfowl (flock size: 101-500)	310 (4)	23 (7; 4.7-10.9)	0 (0)
Commercial water fowl (flock size: >500)	141 (2)	8 (6; 2.4-10.8)	1 (1; 0.01-3)
Health status of waterfowl			
Apparently healthy	7801 (98)	492 (6; 5.6-6.8)	210 (3; 2.3-3)
Sick	194 (2)	5 (3; 0.8-5.9)	2 (1; 0.1-3.6)
Dead	2 (1)	0 (0)	0 (0)
Surveillance sites			
Rajshahi	2219 (28)	140 (6; 5.3-7.4)	42 (2; 1.3-2.5)
Netrokona	2219 (28)	124 (6; 4.6-6.6)	30 (1; 0.9-1.9)
Chittagong	2179 (27)	163 (7; 6.4-8.6)	98 (4; 3.6-5.4)
Dinajpur	1380 (17)	70 (5; 3.9-6.3)	42 (3; 2.2-4)
Sampling year			
2007	259 (3)	14 (5; 2.9-8.9)	4 (2; 0.4-3.9)
2008	718 (9)	18 (3; 1.4-3.9)	3 (1; 0.08-1.2)
2009	720 (9)	27 (4; 2.4-5.4)	3 (1; 0.08-1.2)
2010	720 (9)	43 (6; 4.3-7.9)	4 (1; 0.1-1.4)
2011	960 (12)	68 (7; 5.5-8.8)	39 (4; 2.9-5.5)
2012	960 (12)	63 (7; 5-8.3)	39 (4; 2.9-5.5)
2013	920 (12)	60 (7; 5-8.3)	30 (3; 2.2-4.6)
2014	960 (12)	41 (4; 3-5.7)	5 (1; 0.1-1.2)
2015	940 (12)	50 (5; 3.9-6.9)	23 (2; 1.5-3.6)
2016	840 (11)	113 (13; 11.2-15.9)	62 (7; 5.7-9.3)

Sampling season			
Summer (March-June)	2560 (32)	93 (4; 2.9-4.4)	56 (2; 1.6-2.8)
Monsoon (July-October)	2779 (35)	180 (6; 5.5-7.4)	78 (3; 2.2-3.4)
Winter (November-February)	2658 (33)	224 (8; 7.3-9.5)	78 (3; 2.3-3.6)

Figure 2.2. Month wise avian influenza virus detection in waterfowl from live bird markets (October 2007 to December 2016)

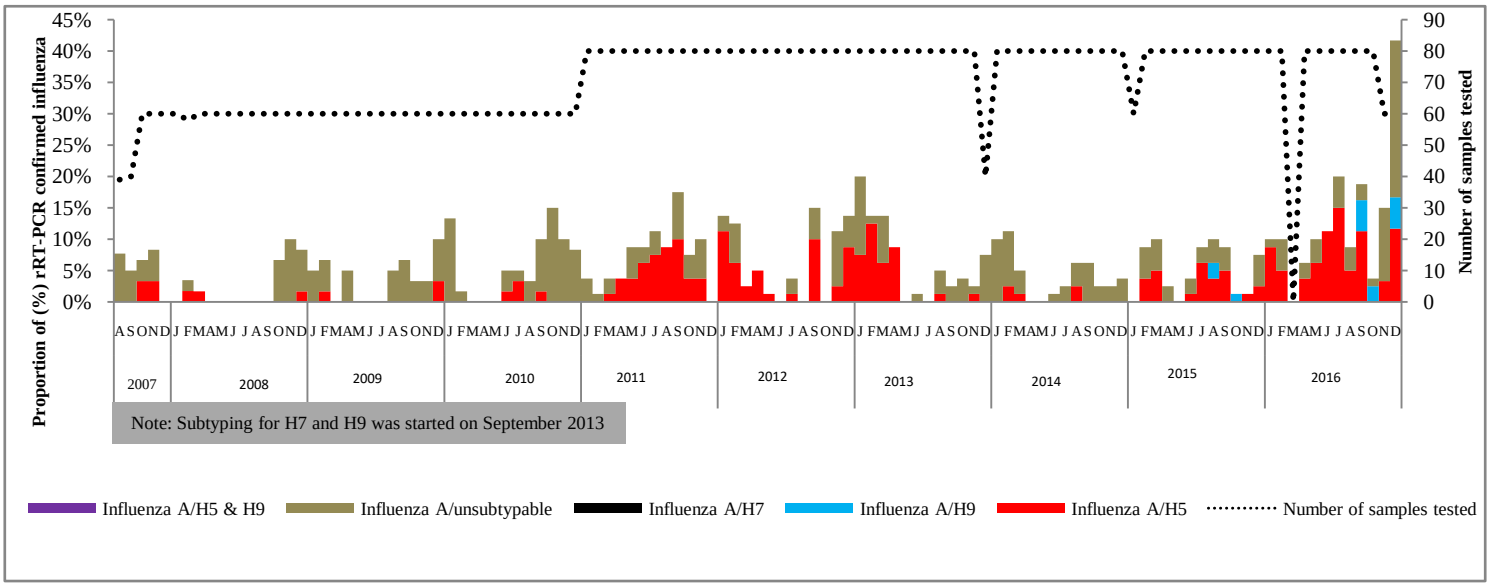


Table 2.3 Univariable and multivariable logistic regression analysis of the associated factors for avian influenza A (N=497) in waterfowl sold at LBMs, Bangladesh, 2007-2016 (Model-1)

Factors associated with influenza A infection	**Univariable analysis**			**Multivariable analysis**		
	PR	**95% CI**	***p*-value**	**PR**	**95% CI**	***p*-value**
Types of waterfowl						
Geese	Ref.			Ref.		
Duck	3.4	2.2-5.4	<0.001	3.1	1.9-4.9	<0.001
Age of waterfowl						
Juvenile	Ref.					
Adult	1.2	0.9-1.6	0.234			
Influenza A seasonality						
Summer (March-June)	Ref.			Ref.		
Monsoon (July-October)	1.8	1.4-2.3	<0.001	1.7	1.3-2.2	<0.001
Winter (November-February)	2.3	1.8-2.9	<0.001	2.2	1.7-2.8	<0.001
Husbandry practice						
Backyard (1-100 poultry)	Ref.					
Small scale (101-500 poultry)	1.2	0.8-1.8	0.372			
Commercial (>500 poultry)	0.9	0.5-1.8	0.807			
Health status						
Apparently healthy	Ref.					
Sick	0.4	0.2-0.9	0.044			
Dead	undefined	-	-			

2.3.2. Status of Avian Influenza Viruses Circulation in Commercial Chicken and Associated Factors

A total of 5,400 commercial chickens were sampled over the surveillance period (2007-2011) in LBMs. Broiler chicken was the major type of commercial chicken (71%). All commercial chickens were adult and 99% were apparently healthy (Table 2.4).

Among the tested commercial chickens, 3% commercial chickens had detectable RNA for influenza A virus of which 1% commercial chickens had detectable RNA for H5 subtype. Among commercial chicken, Cobb chickens (a breed of broiler chicken) were more likely to be positive for AI A viruses than other broiler, layer and breeder chickens. The commercial chicken that were sampled during winter and monsoon seasons were more likely to test positive for influenza A viruses compared to the commercial chicken sampled during summer (Table 2.4). Avian influenza viruses were more commonly detected in 2016 than previous years. The difference for year wise avian influenza detection was statistically significant ($p < 0.001$). There was no significant difference for AI A/H5 in terms of surveillance sites. Avian influenza A/H5 was more frequently detected in dead chicken than healthy and sick chicken. In the final multivariable analysis model, sources of the commercial chicken and seasonality were significantly associated with avian influenza (Table 2.5).

Table 2.4. Demographics and detection of influenza virus type A and H5 subtype among commercial chicken from live bird markets, Bangladesh, September 2013-December 2016

Characteristics	Total no of samples n (%)	rRT-PCR positive sample number	
		Influenza virus A (all subtypes) n (%; 95% CI)	H5 Subtype n (%; 95% CI)
Production type			
Broiler	3802 (70)	124 (3; 2.7-3.8)	12 (1; 0.1-0.5)
Layer	1162 (22)	48 (4; 3-5.4)	15 (1; 0.7-2.1)
Breeder (Spent parent stock)	420 (8)	7 (2; 0.6-3.4)	0 (0)
Cobb (a specific broiler breed)	16 (1)	4 (25; 7.2-52.3)	0 (0)
Age of commercial chicken			
Adult	5400 (100)	183 (3, 2.9-3.9)	27 (1; 0.3-0.7)
Sources of commercial chicken			
Same sub-districts of markets	1140 (21)	20 (2; 1-2.6)	0 (0)
Other sub-districts	4260 (79)	163 (4; 3.2-4.4)	27 (1; 0.4-0.9)
Health status of sampled commercial chicken			
Apparently healthy	5356 (99)	172 (3; 2.7-3.7)	24 (1; 0.2-0.6)
Sick	7 (1)	0 (0)	0 (0)
Dead	37 (1)	11 (30; 15.8-46.9)	3 (8; 1.7-21.9)
Surveillance sites			
Chittagong	17 (1)	4 (23; 6.8-49.8)	0 (0)
Dhaka	3020 (56)	90 (3; 2.4-3.6)	18 (1; 0.3-0.9)
Dinajpur	185 (3)	2 (1; 0.1-3.8)	0 (0)
Gazipur	1120 (21)	69 (6; 4.8-7.7)	9 (1; 0.3-1.5)
Kishoreganj	540 (10)	5 (1; 0.3-2.1)	0 (0)
Netrokona	180 (3)	2 (1; 0.1-3.9)	0 (0)
Rajshahi	180 (3)	11 (6; 3-10.6)	0 (0)
Sampling year			
2013	570 (11)	13 (2; 1.2-3.8)	3 (1; 0.1-1.5)
2014	1740 (32)	31 (2; 1.2-2.5)	6 (1, 0.1-0.7)
2015	1715 (32)	68 (4; 3-4.9)	6 (1; 0.1-0.7)
2016	1375 (25)	71 (5; 4-6.4)	12 (1; 0.4-1.5)
Sampling season			
Summer (March-June)	1595 (30)	36 (2; 1.5-3.1)	5 (1; 0.1-0.7)
Monsoon (July-October)	2030 (38)	84 (4; 3.3-5)	11 (1; 0.2-0.9)
Winter (November-February)	1775 (33)	63 (4; 2.7-4.5)	11 (1; 0.3-1.1)

Figure 2.3. Month wise avian influenza virus detection in commercial chicken from live bird markets (September 2013 to December 2016)

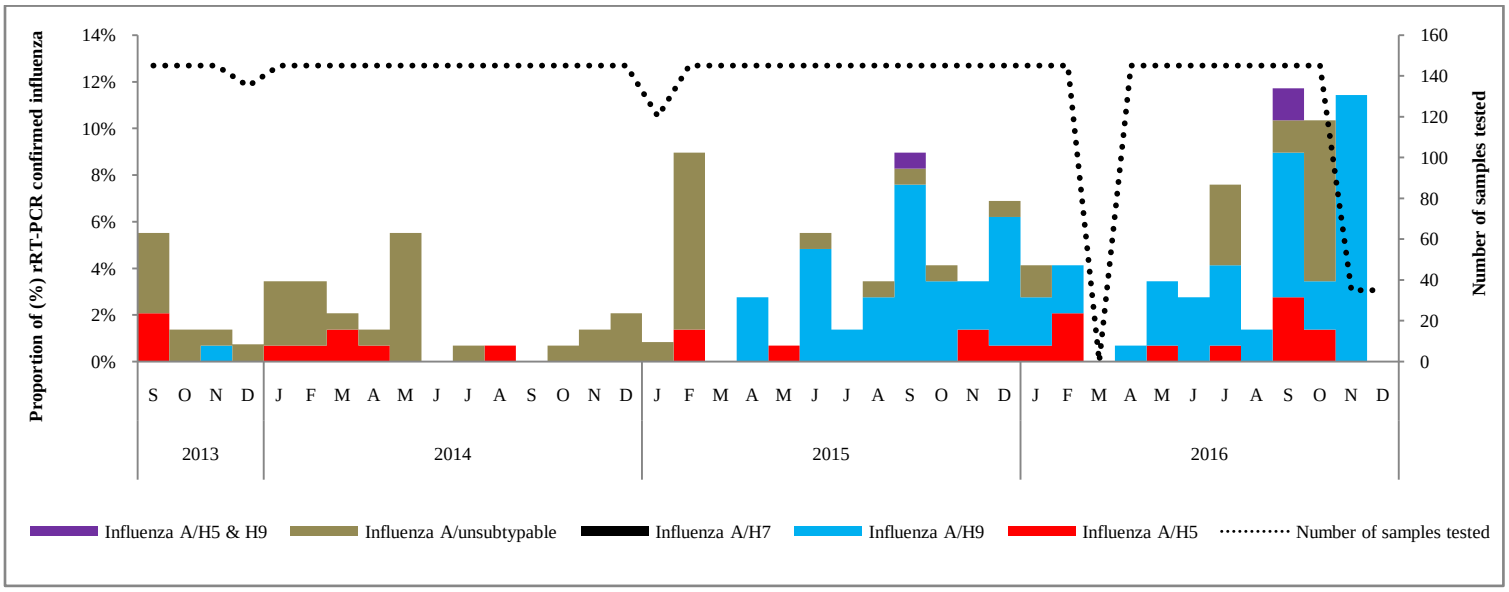


Table 2.5. Univariable and multivariable logistic regression analysis of the associated factors for avian influenza A (N=183) in commercial chicken sold at LBMs, Bangladesh, 2013-2016 (Model-2)

Factors associated with influenza A infection	Univariable analysis			Multivariable analysis		
	PR	95% CI	*p*-value	PR	95% CI	*p*-value
Types of commercial chicken						
Broiler	Ref.					
Layer	1.2	0.9-1.8	0.156			
Breeder (Spent parent stock)	0.5	0.2-1.1	0.081			
Cobb	7.6	3.2-18.2	<0.001			
Health status	1.2					
Apparently healthy	Ref.					
Sick	undefined	-	-			
Dead	9.2	5.5-15.5	<0.001			
Influenza A seasonality						
Summer (March-June)	Ref.			Ref.		
Monsoon (July-October)	1.8	1.2-2.7	0.002	1.8	1.2-2.6	0.003
Winter (November-February)	1.6	1.1-2.3	0.028	1.6	1.1-2.4	0.028
Sources of commercial chicken						
Same sub-districts of markets	Ref.			Ref.		
Other sub-districts	2.2	1.4-3.4	0.001	2.2	1.4-3.4	0.001

2.3.3. Status of Avian Influenza Viruses Circulation in Backyard Chicken and Associated Factors

During September 2013 to December 2016, a total of 1,860 backyard chickens were sampled in selected LBMs. Two thirds of the backyard chickens were adult and 90% of them were apparently healthy. The mean and median backyard poultry flocks size was 21 and 16 respectively (range: 1-440). We identified that 2% of backyard chicken had detectable RNA for influenza A virus of which 1% had H5 subtype (Table 2.6). More detection of influenza A/H5 was recorded in winter and in 2014 (Figure 2.4). However, the difference for year wise avian influenza detection was statistically insignificant (p >0.357). The backyard chicken that were sampled during winter were more likely to test positive for influenza A viruses compared to the backyard chicken sampled during summer or monsoon (Table 2.7). No significant difference for AI A/H5 was found among different surveillance sites. However, AI A/H5 was more commonly detected in sick chicken than healthy chicken. The poultry mortality history of the past seven days of sample collection was less than 1% over the surveillance period. In the final multivariable analysis model, health condition of backyard poultry and seasonality was significantly associated with avian influenza (Table 2.6).

Table 2.6. Demographics and detection of influenza virus type A and H5 subtype among backyard chicken from live bird markets, Bangladesh, September 2013 - December 2016

Characteristics	Total no of samples n (%)	rRT-PCR positive sample number	
		Influenza virus A (all subtypes) n (%; 95% CI)	H5 Subtype n (%; 95% CI)
Age of backyard chicken			
Juvenile	611 (33)	9 (1; 0.6-2.7)	6 (1; 0.3-2.1)
Adult	1249 (67)	24 (2; 1.2-2.8)	12 (1; 0.4-1.6)
Flock size			
≥21 poultry	1245 (67)	18 (1; 0.8-2.2)	9 (1; 0.3-1.3)
≤22 poultry	615 (33)	15 (2; 1.3-3.9)	9 (1; 0.6-2.7)
Health status of sampled backyard chicken			
Apparently healthy	1679 (90)	23 (1; 0.8-2)	11 (1; 0.3-1.1)
Sick	179 (10)	10 (6; 2.7-10)	7 (4; 1.5-7.8)
Dead	2 (1)	0 (0)	0 (0)
Surveillance sites			
Chittagong	370 (20)	7 (2; 0.7-3.8)	4 (1; 0.2-2.7)
Jessore	360 (20)	5 (1; 0.4-3.2)	0 (0)
Netrokona	390 (21)	10 (3; 1.2-4.6)	7 (2; 0.7-3.6)
Bogra	360 (19)	5 (1; 0.4-3.2)	2 (1; 0.06-1.9)
Faridpur	360 (19)	6 (2; 0.6-3.5)	5 (1; 0.4-3.2)
Rajshahi	20 (1)	0 (0)	0 (0)

Sampling year			
2013	170 (9)	2 (1; 0.1-4.1)	2 (1; 0.1-4.1)
2014	600 (32)	15 (3; 1.4-4)	11 (2; 0.9-3.2)
2015	580 (31)	7 (1; 0.4-2.4)	4 (1; 0.004-0.9)
2016	510 (27)	9 (2; 0.8-3.3)	1 (1; 0.004-1)
Sampling season			
Summer (March-June)	550 (30)	4 (1; 0.1-1.8)	4 (1; 0.1-1.8)
Monsoon (July-October)	700 (38)	11 (2; 0.7-2.7)	4 (1; 0.1-1.4)
Winter (November-February)	610 (33)	18 (3; 1.7-4.6)	10 (2; 0.7-2.9)

Figure 2.4. Summary of influenza A surveillance in backyard chicken from live bird markets (September 2013 to December 2016)

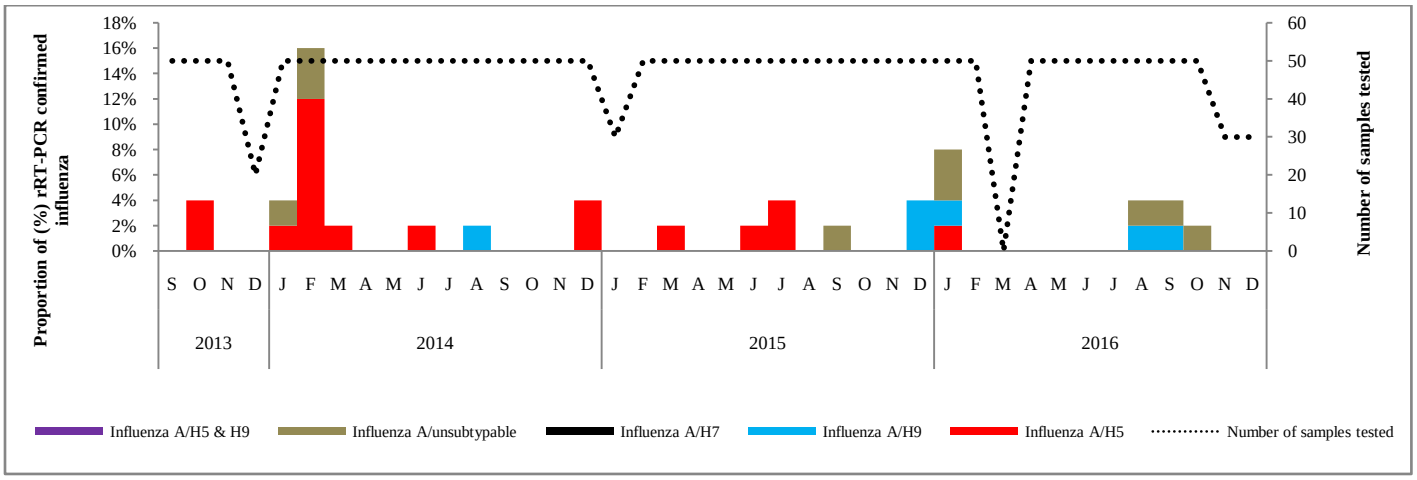


Table 2.7. Univariable and multivariable logistic regression analysis of the associated factors for avian influenza A (N=33) in backyard chicken sold at LBMs, Bangladesh, 2013-2016 (Model-3)

Factors associated with influenza A infection	**Univariable analysis**			**Multivariable analysis**		
	PR	**95% CI**	***p*-value**	**PR**	**95% CI**	***p*-value**
Flock size of backyard poultry						
≥21 poultry	Ref.			Ref.		
≤22 poultry	1.7	0.8-3.3	0.131			
Age of backyard chicken						
Juvenile	Ref.					
Adult	1.3	0.6-2.8	0.493			
Health status						
Apparently healthy	Ref.			Ref.		
Sick	4.1	1.9-8.4	<0.001	3.8	1.8-8	<0.001
Dead	undefined	-	-	undefined	-	-
Influenza A seasonality						
Summer (March-June)	Ref.			Ref.		
Monsoon (July-October)	2.2	0.7-6.7	0.185	2.2	0.8-7.7	0.125
Winter (November-February)	4.1	1.4-11.9	0.011	3.8	1.3-11.1	0.015

2.3.4. Status of Avian Influenza Viruses Circulation in Environmental Samples in LBMs

From May 2009 to December 2016, a total of 1,920 pooled environmental samples were obtained and tested for AI A virus and H5 subtype. Among the tested samples, 29% were positive for influenza A virus of which 10% tested positive for influenza A/H5 subtype (Table 2.8). Seventy one percent of the environmental pooled samples were collected from urban LBMs.

Environmental samples collected from urban LBMs were more positive for AI A/H5 than the rural or peri-urban LBMs specimens. The highest proportion (33%) of AI A positive/H5 in environmental samples was detected in 2011 (Figure 2.5). The difference for year wise avian influenza detection was statistically significant ($p < 0.001$). Environmental samples collected during colder months (October –March) were more positive than other season. However, location of LBM was significantly associated with avian influenza in multivariable analysis model (Table 2.9).

Table 2.8. Detection of influenza virus type A and H5 subtype in environmental specimens from live bird markets in Bangladesh, May 2009 - December 2016

Characteristics	Total no of samples n (%)	rRT-PCR positive sample number	
		Influenza virus A (all subtypes) n (%; 95% CI)	H5 Subtype n (%; 95% CI)
Type of live bird market			
Peri-urban	549 (29)	102 (19; 15.4-22)	25 (5; 2.9-6.6)
Urban	1,371 (71)	459 (33; 30.9-36)	169 (12; 10.6-14.1)
Seasons in which sampling conducted			
Summer (March-June)	626 (33)	159 (25; 22-29)	65 (10; 8.1-13)
Monsoon (July-October)	677 (35)	215 (32; 28.2-35.4)	46 (7; 5-8.9)
Winter (November-February)	617 (32)	187 (30; 26.7-34.1)	83 (13; 10.8-16.4)
Years in which sampling conducted			
2009	45 (2)	26 (58; 42.1-72.3)	11 (24; 12.8-39.5)
2010	132 (7)	79 (60; 50.9-68.2)	19 (14; 8.8-21.5)
2011	215 (11)	113 (53; 45.6-59.3)	71 (33; 26.7-39.7)
2012	252 (13)	56 (22; 17.2-27.8)	34 (13; 9.5-18.3)
2013	274 (14)	57 (21; 16.1-26)	23 (8; 5.3-12.3)
2014	348 (18)	37 (11; 7.5-14.3)	6 (2; 0.6-3.7)
2015	344 (18)	83 (24; 19.7-29)	4 (1; 0.3-2.9)
2016	310 (16)	110 (35; 30.1-41)	26 (8; 5.5-12)

Figure 2.5. Summary of influenza A surveillance in pooled environmental samples from live bird markets (May 2009 - December 2016)

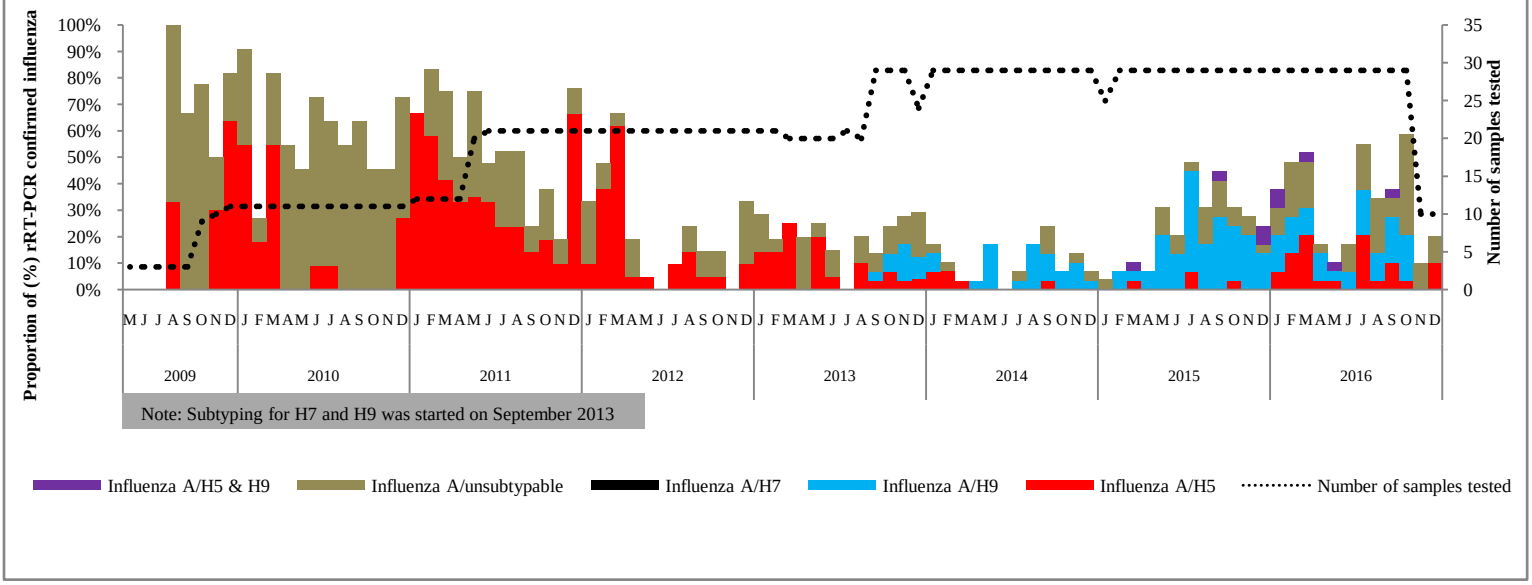


Table 2.9. Univariable and multivariable logistic regression analysis of the associated factors for avian influenza A (N=561) in environmental specimens collected from LBMs, Bangladesh, 2009-2016 (Model-4)

Factors associated with influenza A infection	Univariable analysis			Multivariable analysis		
	PR	95% CI	*p*-value	PR	95% CI	*p*-value
Type of live bird market						
Peri-urban	Ref.			Ref.		
Urban	1.8	1.5-2.2	<0.001	1.8	1.5-2.2	<0.001
Seasons in which sampling conducted						
Summer (March-June)	Ref.					
Monsoon (July-October)	1.3	1.1-1.5	0.012			
Winter (November-February)	1.2	0.9-1.4	0.054			

2.3.5. Avian influenza sub-types and sequencing of Avian Influenza Isolates at CDC Laboratory

Multiple AI subtypes in different combinations were detected in different poultry species in the current study. H5N1 (HPAI), H9N2 (LPAI), H11N3 (LPAI), H4N6 (LPAI), and H1N1 (LPAI) were commonly detected subtypes. Other LPAI subtypes were H1N3, H2N4, H3N2, H3N6, H3N8, H4N2, H5N2, H6N1, H6N7, and H7N9 (Table 2.10). Gene sequences for both HPAI and LPAI viruses were submitted to GISAID (Global Initiative on Sharing All Influenza Data, http://platform.gisaid.org). GISAID accession numbers include EPI448024-448111, EP448120-448279, EP448883-448924, EP353364, EP353365, EP353370, EP353372, EP353379, EP353381, EP314772-314779, EP219467-219474, EP460194-460201, EPI448288-95, EPI448280-87, EPI457484-91, EPI540152-507, EPI484574-77, EPI484579-80, EPI540527-44.

Table 2.10. Information about LPAI viruses isolated by subtype from poultry and environment of LBM under avian influenza surveillance

Virus name	Date of sample collection (mm/dd/yy)	Subtype	Species
A/duck/Bangladesh/1687/2010	7/16/2010	H1N1	Duck
A/duck/Bangladesh/31687/2010	7/11/2010	H1N1	Duck
A/duck/Bangladesh/1592/2010	1/20/2010	H1N1	Duck
A/duck/Bangladesh/1352/2009	1/28/2009	H1N1	Duck
A/duck/Bangladesh/1584/2010	1/20/2010	H1N3	Duck
A/duck/Bangladesh/1822/2011	1/19/2011	H3N2	Duck
A/duck/Bangladesh/1025/2011	2/21/2011	H3N2	Duck
A/duck/Bangladesh/1772/2010	11/12/2010	H3N2	Duck
A/duck/Bangladesh/1798/2010	11/10/2010	H3N6	Duck
A/duck/Bangladesh/1800/2010	11/10/2010	H3N6	Duck
A/duck/Bangladesh/1574/2009	12/23/2009	H3N8	Duck
A/duck/Bangladesh/1575/2009	12/23/2009	H3N8	Duck
A/duck/Bangladesh/1576/2009	12/23/2009	H3N8	Duck
A/duck/Bangladesh/1745/2010	10/17/2010	H4N2	Duck
A/duck/Bangladesh/1746/2010	10/17/2010	H4N2	Duck
A/duck/Bangladesh/1766/2010	10/27/2010	H4N6	Duck
A/duck/Bangladesh/1521/2009	10/21/2009	H4N6	Duck
A/duck/Bangladesh/1283/2008	12/21/2008	H4N6	Duck
A/duck/Bangladesh/1783/2010	11/10/2010	H4N6	Duck
A/duck/Bangladesh/1784/2010	11/10/2010	H4N6	Duck

A/duck/Bangladesh/1559/2009	12/18/2009	H5N2	Duck
A/duck/Bangladesh/1293/2008	11/21/2008	H6N1	Duck
A/waterfowl/Bangladesh/12301/2013	1/23/2013	H6N7	Waterfowl
A/environment/Bangladesh/1008/2010	9/23/2010	H7N9	LBM environment
A/environment/Bangladesh/917/2012	3/14/2012	H7N9	LBM environment
A/environment/Bangladesh/100/2010	5/25/2010	H9N2	LBM environment
A/environment/Bangladesh/124/2010	07/19/2010	H9N2	LBM environment
A/environment/Bangladesh/155/2010	10/20/2010	H9N2	LBM environment
A/environment/Bangladesh/177/2010	12/12/2010	H9N2	LBM environment
A/duck/Bangladesh/1727/2010	9/17/2010	H11N3	Duck
A/duck/Bangladesh/1728/2010	9/17/2010	H11N3	Duck
A/duck/Bangladesh/1729/2010	9/17/2010	H11N3	Duck
A/duck/Bangladesh/1753/2010	9/15/2010	H11N3	Duck
A/duck/Bangladesh/1595/2010	1/20/2010	H11N3	Duck
A/duck/Bangladesh/1578/2009	12/23/2009	H11N3	Duck
A/duck/Bangladesh/1051/2007	10/31/2007	H11N3	Duck
A/duck/Bangladesh/1052/2007	10/31/2007	H11N3	Duck
A/environment/Bangladesh/1002/2010	3/14/2010	H11N3	LBM environment

2.3.6. Different Clades of HPAI H5N1

HPAI H5N1 Clade 2.2.2 was recognized from domestic chickens sampled in 2010. Clade 2.3.2.1 was more frequent in domestic chicken, waterfowl and environmental samples in 2011 and 2012. Clade 2.3.4 was recognized in few chicken samples collected in 2011 (Table 2.11).

Table 2.11. Information about HPAI (H5N1) viruses isolated from poultry and environment of LBM under avian influenza surveillance

Virus name	Date of sample collection (mm/dd/yy)	HA clade	Species
A/chicken/Bangladesh/0912/2010	1/4/2010	2.2.2	Domestic chicken
A/chicken/Bangladesh/1012/2010	1/4/2010	2.2.2	Domestic chicken
A/chicken/Bangladesh/0411/2010	1/12/2010	2.2.2	Domestic chicken
A/poultry/Bangladesh/11255-C/2011	2/7/2011	2.2.2	Poultry
A/chicken/Bangladesh/31289-1/2011	2/20/2011	2.2.2	Domestic chicken
A/chicken/Bangladesh/11303/2011	2/4/2011	2.3.2.1	Domestic chicken
A/duck/Bangladesh/1849/2011	3/20/2011	2.3.2.1	Duck
A/chicken/Bangladesh/3072/2011	5/23/2011	2.3.2.1	Domestic chicken
A/chicken/Bangladesh/3075/2011	5/24/2011	2.3.2.1	Domestic chicken
A/environment/Bangladesh/1017/2011	5/29/2011	2.3.2.1	LBM Environment
A/waterfowl/Bangladesh/33025/2011	6/29/2011	2.3.2.1	Waterfowl
A/goose/Bangladesh/4051T/2011	7/1/2011	2.3.2.1	Goose
A/chicken/Bangladesh/4058/2011	7/14/2011	2.3.2.1	Domestic chicken
A/duck/Bangladesh/4059T/2011	7/14/2011	2.3.2.1	Duck
A/chicken/Bangladesh/4070T/2011	7/15/2011	2.3.2.1	Domestic chicken
A/waterfowl/Bangladesh/31935/2011	7/17/2011	2.3.2.1	Waterfowl
A/duck/Bangladesh/4117T/2011	7/24/2011	2.3.2.1	Duck
A/duck/Bangladesh/4120T/2011	7/24/2011	2.3.2.1	Duck
A/duck/Bangladesh/4124T/2011	7/24/2011	2.3.2.1	Duck
A/environment/Bangladesh/1018/2011	9/29/2011	2.3.2.1	LBM Environment
A/environment/Bangladesh/1011/2011	12/28/2011	2.3.2.1	LBM Environment
A/environment/Bangladesh/1017-1/2011	12/30/2011	2.3.2.1	LBM Environment
A/chicken/Bangladesh/42010/2012	1/8/2012	2.3.2.1	Domestic chicken
A/duck/Bangladesh/32077/2012	2/20/2012	2.3.2.1	Duck
A/environment/Bangladesh/1019-G/2012	2/28/2012	2.3.2.1	LBM Environment
A/chicken/Bangladesh/3012/2011	2/19/2011	2.3.4.2	Domestic chicken
A/chicken/Bangladesh/11RS-1984-30/2011	6/15/2011	2.3.4.2	Domestic chicken

2.3.7. Phylogenetic analysis, geo-temporal relationships and genotyping of viruses

The sequences of 5 H5N1 viruses determined from the samples collected between 2010 and early 2011 from poultry were clustered with previously described clade 2.2.2 viruses (Figure 2.6). HA gene sequences of H5N1 revealed that viruses isolated from samples collected in 2010 formed a cluster with the sequences of H5N1 viruses isolated in poultry in Bhutan (Figure 2.6). The sequences of H5N1 viruses isolated from India were also grouped in same larger cluster. There was no evidence of genotypic reassortment for clade 2.2.2 viruses in this study (Figure 2.6).

Phylogenetic tree analysis revealed the sequences of 30 H5N1 viruses collected during and after 2011 were closely clustered with clade 2.3.2.1 Hubei-like lineage (Figure 2.6). Heterogenicity among these clade 2.3.2.1 viruses from Bangladeshi isolates indicates that multiple introductions occurred into the country or that circulation of viruses occurred for a sustained period of time (Figure 2.6). The HA sequences of clade 2.3.2.1 viruses revealed these isolates were close congeners to the sequences of H5N1 viruses isolated from India (A/chicken/India/CA301/2011) and Nepal (A/chicken/Nepal/T1P/2012) (Figure 2.6). This group of viruses also shared a common node with Hubei-like viruses from Vietnam collected from 2011 to 2012 (Figure 2.6).

Two H5N1 viruses (A/chicken/Bangladesh/3012/2011 and A/chicken/Bangladesh/11RS-1984-30/2011; Figure 2.6) collected during 2011 from chicken belonged to clade 2.3.4.2. This group of viruses was grouped in same cluster of viruses that were collected in such as Myanmar, Vietnam, Lao PDR and China (Figure 2.6).

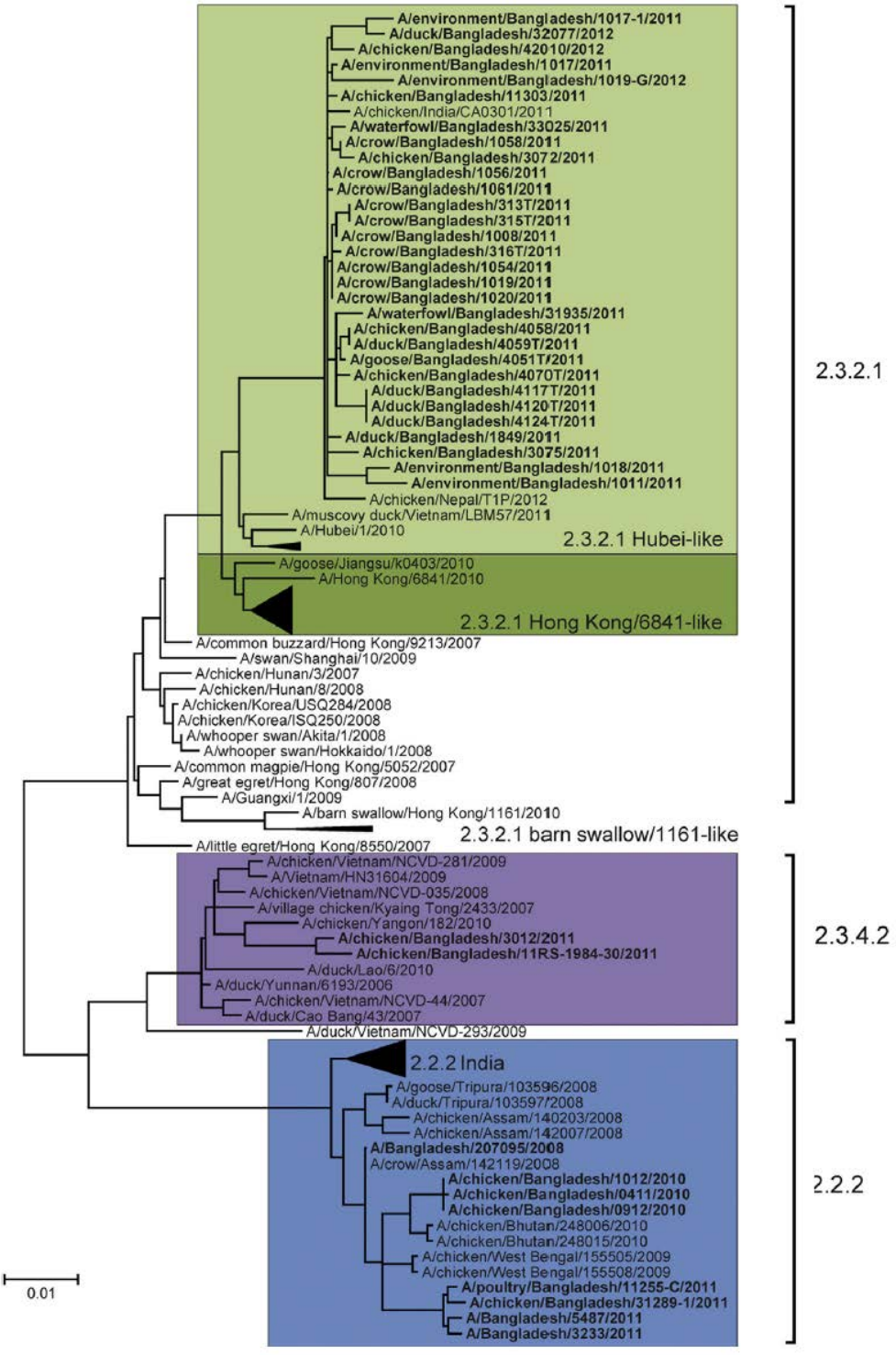


Figure 2.6. Phylogenetic tree of HA gene sequences including Bangladeshi H5N1 viruses. The clades are highlighted with color code, light green for 2.3.2.1 Hubei-like, dark green (Hong Kong/6841-like), purple for clade 2.3.4.2 and blue for clade 2.2.2.

The sequences of hemagglutinin gene segment of subtype H1 viruses were identical to larger clusters of the sequences of Asian viruses (A/duck/Zhejiang/0224-6/2011 [H1N2]), European viruses, and South African viruses (Figure 2.7). The sequences of HAs of H2 viruses were identical to the sequences of the isolates from Europe (Figure 2.8). The sequences of HA gene segments of two LPAI virus isolates (A/duck/Bangladesh/1822/2011 and A/duck/Bangladesh /1025/2011) from sample collected in 2011 were identical to the sequences of their internal and surface protein coding gene segments and both were subtype H3N2 (Figure 2.9). Similarly, the sequences of another genetically identical pair of viruses (A/duck/Bangladesh/1783/2010 and A/duck/Bangladesh/1784/2010) were grouped in the same cluster and both were subtype H4N6 (Figure 2.10). The sequences of subtype H3N6 and H3N8 viruses were clustered in a group with the sequences of South East Asian virus (A/swan/Shimane/227/01 [H3N9]) and other viruses from Korea, China and Siberia (Figure 2.9).

The sequences of HA of all H4N2 and H4N6 viruses were genetically related to H4 viruses from Central and East Asia, Europe and Egypt (Figure 2.10). The sequence of HA of H5N2 virus was clustered in a group with the sequences of central Asian viruses (e.g. A/duck/Mongolia/194/2011 [H5N3]) (Figure 2.11). The sequence of HA gene segment of H6N1 virus was genetically identical to the sequences of European viruses (A/goose/Germany-BB/R1625/2008 (H6)). The sequence of HA gene of H6N7 was genetically related with the sequences of European viruses (Figure 2.12). Subtype H7N9 was isolated from two environmental samples of LBMs in 2010 and 2012. Both the sequences of H7N9 viruses were genetically related to the sequence of a Central Asian virus (A/wild duck/Mongolia/1-241/2008 [H7N9]) and phylogenetically dissimilar to the sequences of Chinese H7N9 viruses determined in 2013–2014 (Figure 2.13). The sequences of H9N2 subtype isolated from 12 samples were genetically similar to the sequences of Bangladeshi and Indian viruses clustered within the larger G1 lineage of H9N2 viruses (Figure 2.14). The sequence of H11N3 subtype isolated from poultry during 2007 was genetically closely related to the sequences of European viruses A/mallard/Netherlands/17/2007 (Figure 2.15). The sequences of HA gene segments of H11N3 collected during 2009 and 2010 were clustered in a group with the sequences of viruses isolated from Japan and China (A/chicken/Nanjing/908/2009 [H11N2]) (Figure 2.15).

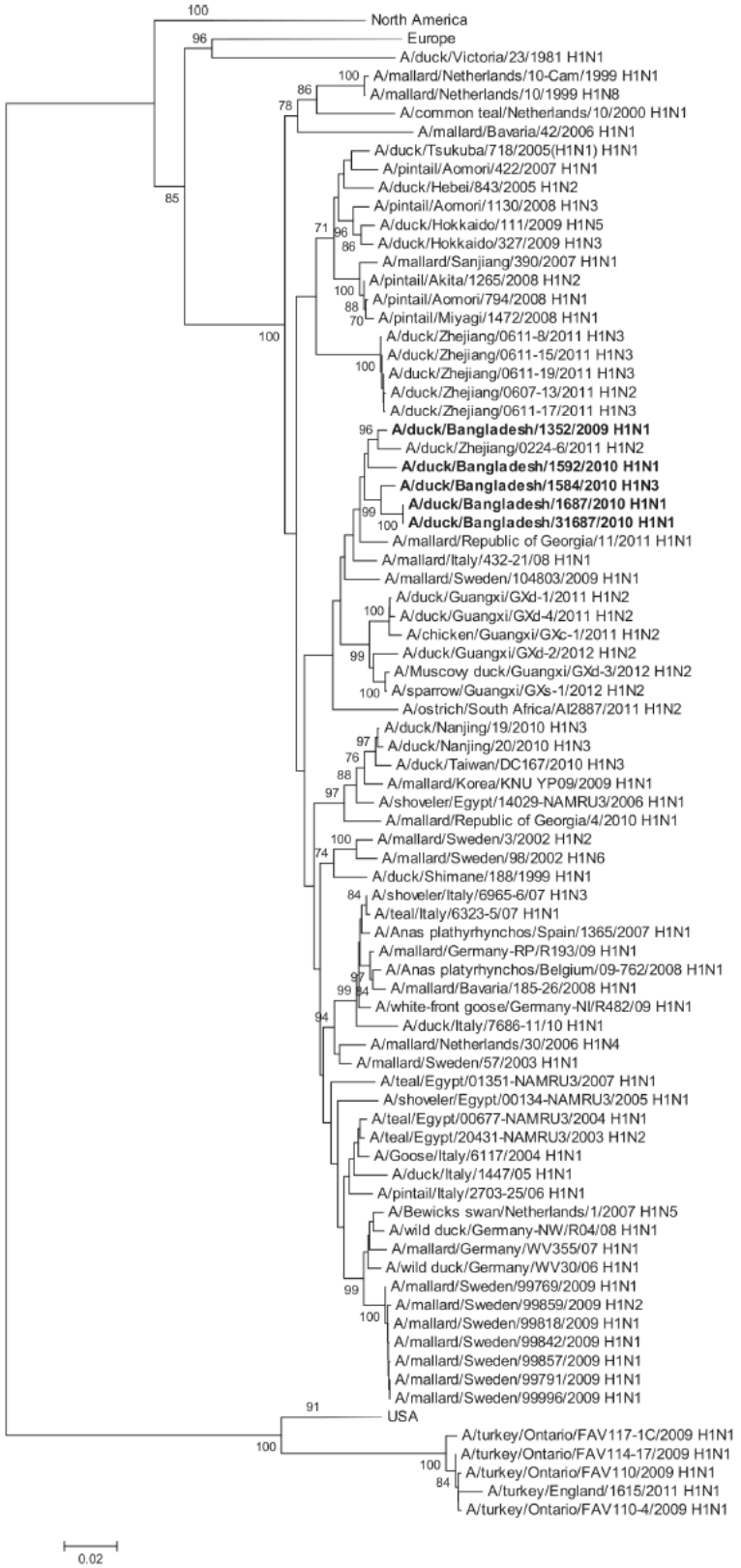


Figure 2.7. Phylogenies of the complete coding hemagglutinin genes for subtype H1

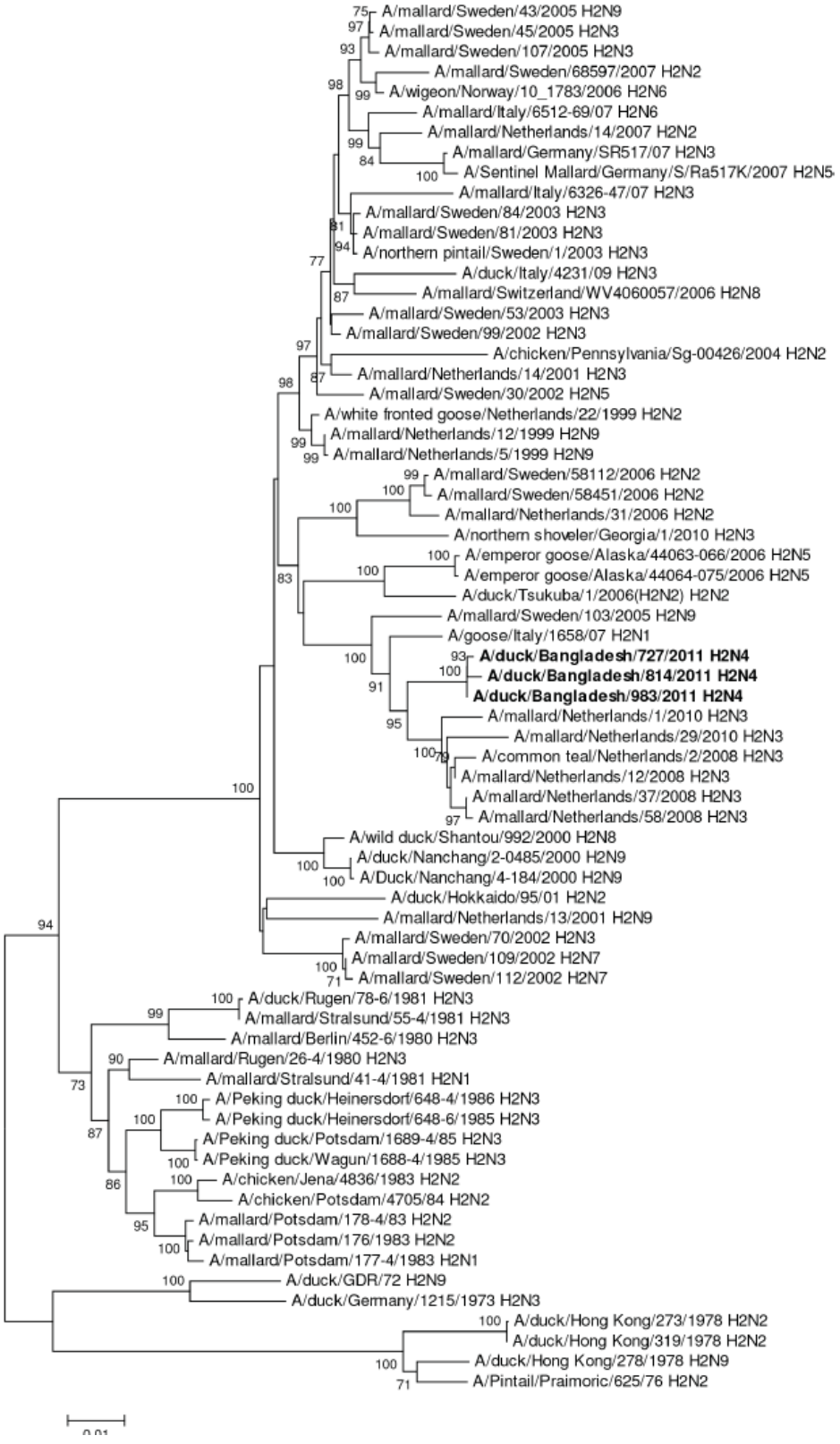


Figure 2.8. Phylogenies of the complete coding hemagglutinin genes for subtype H2

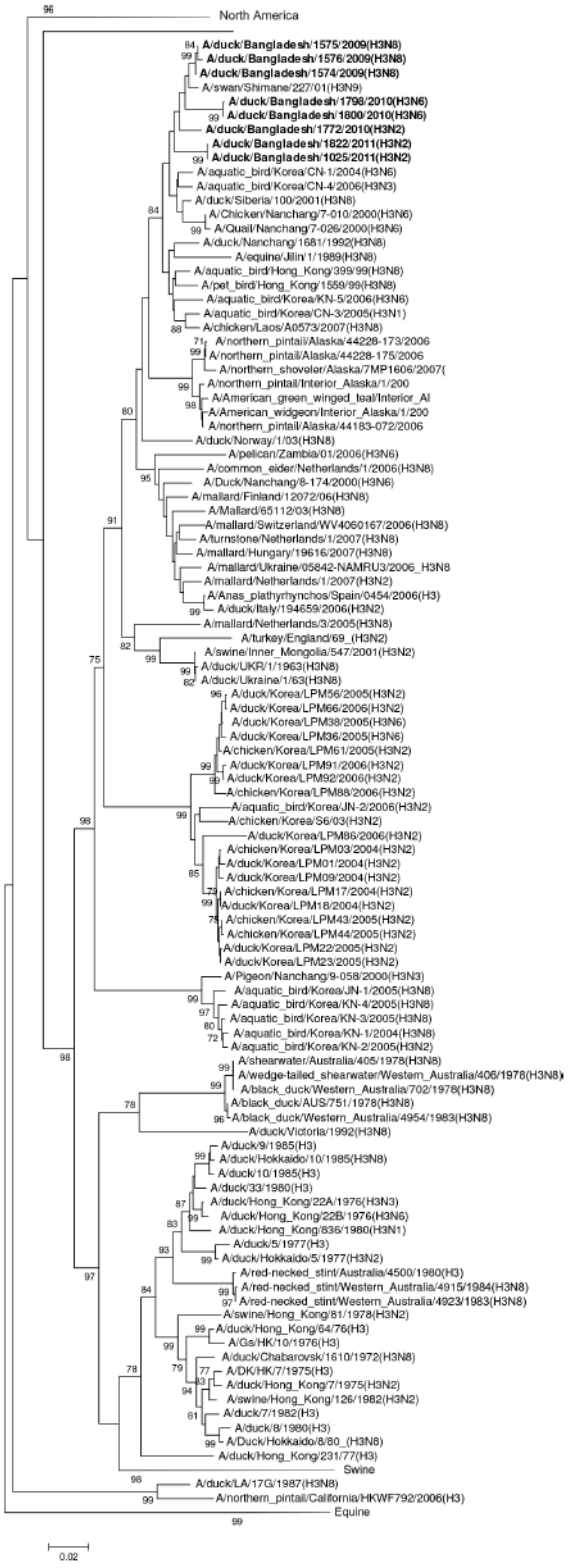


Figure 2.9. Phylogenies of the complete coding hemagglutinin genes for subtype H3

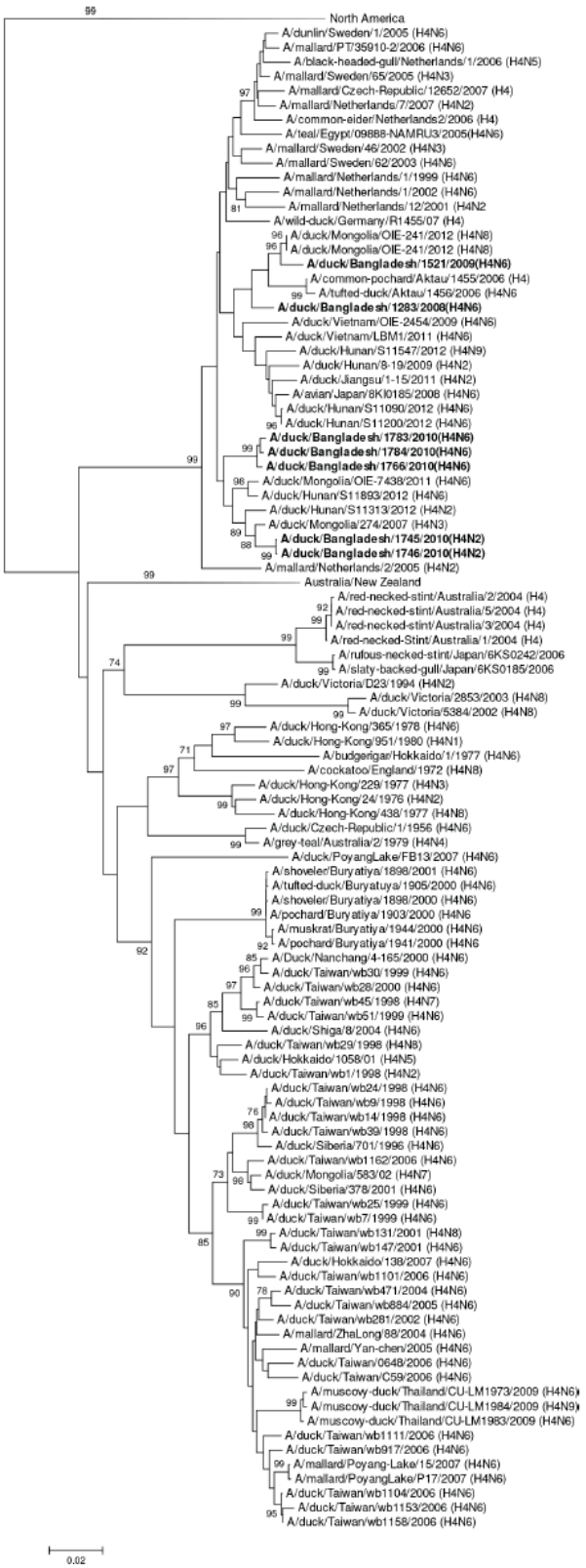


Figure 2.10. Phylogenies of the complete coding hemagglutinin genes for subtype H4

Figure 2.11. Phylogenies of the complete coding hemagglutinin genes for subtype H5

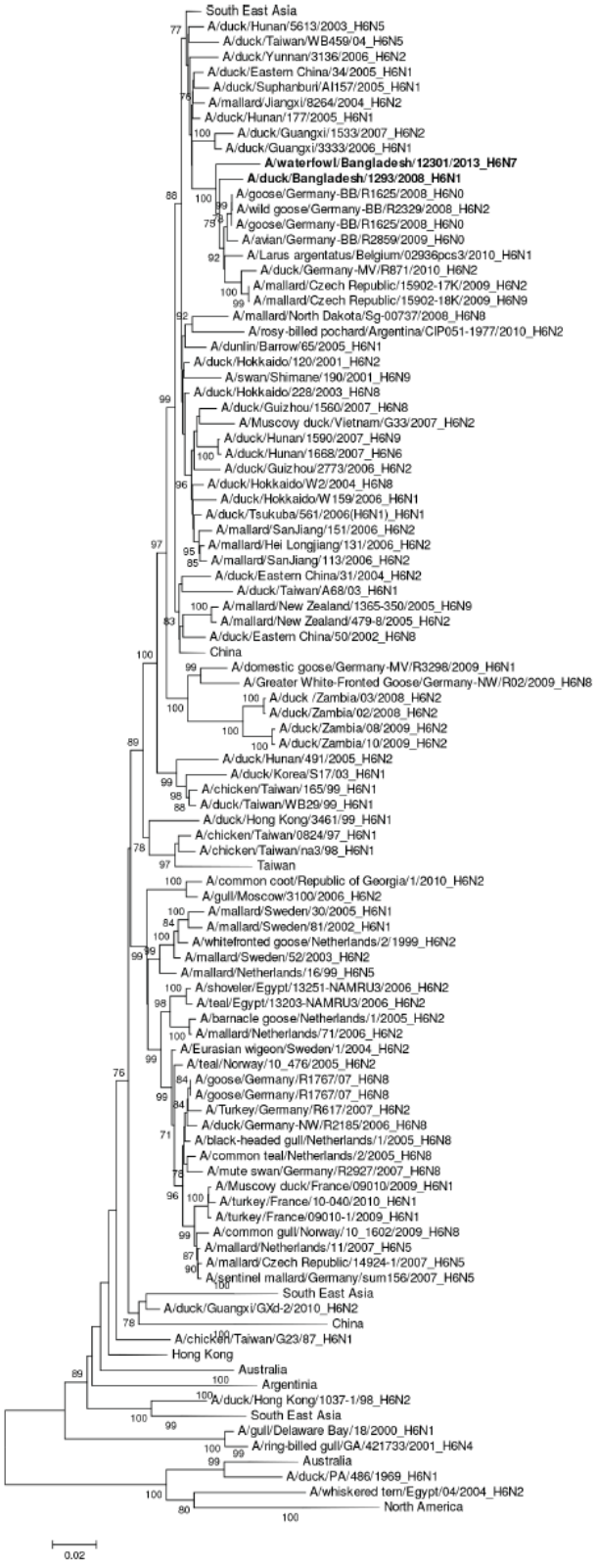


Figure 2.12. Phylogenies of the complete coding hemagglutinin genes for subtype H6

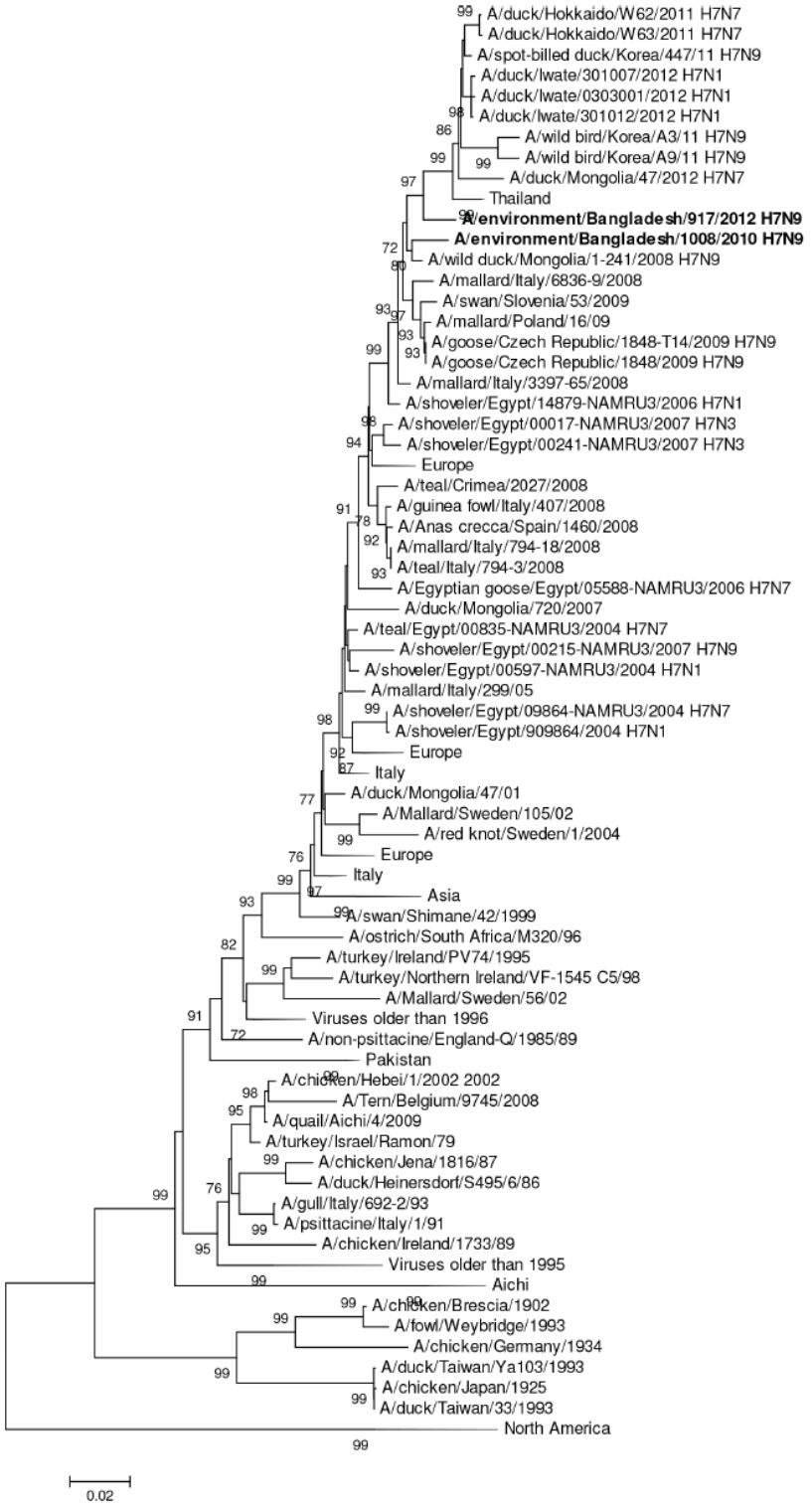


Figure 2.13. Phylogenies of the complete coding hemagglutinin genes for subtype H7

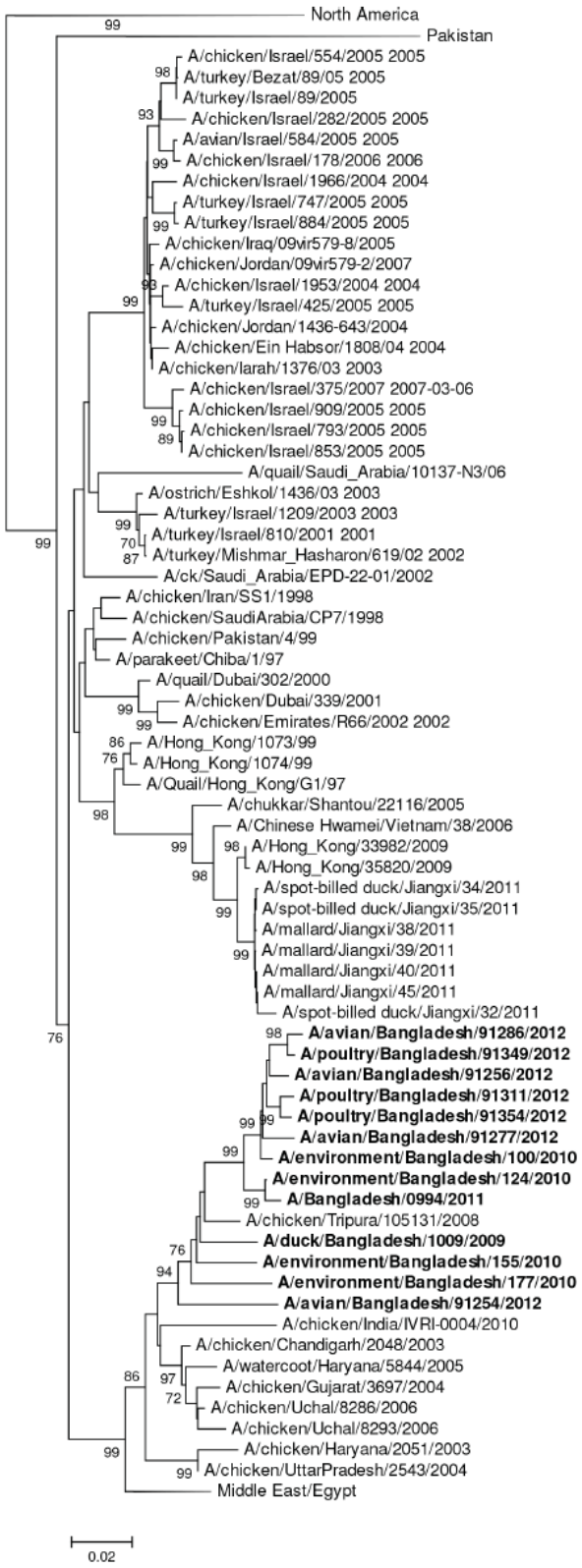


Figure 2.14. Phylogenies of the complete coding hemagglutinin genes for subtype H9

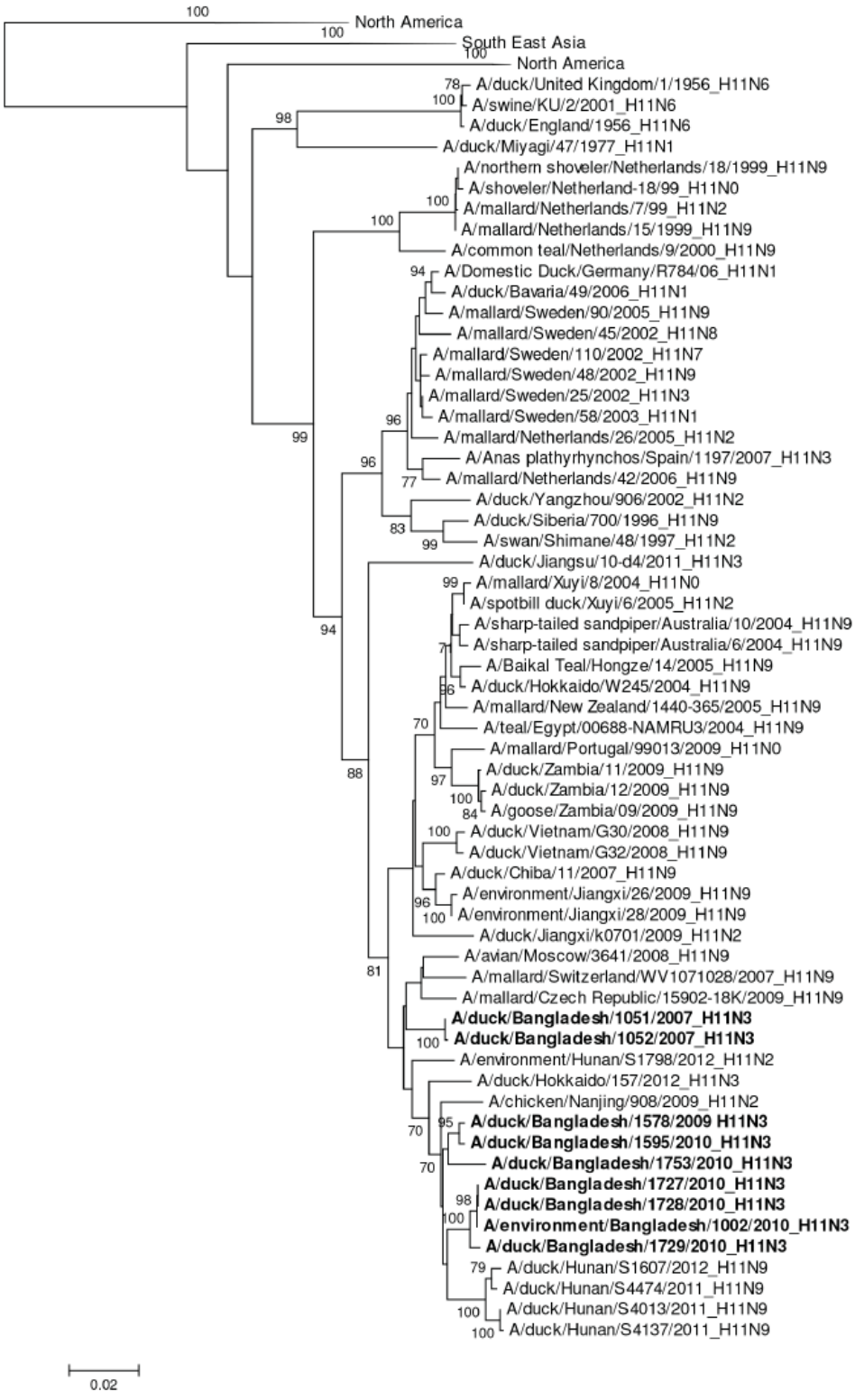


Figure 2.15. Phylogenies of the complete coding hemagglutinin genes for subtype H11

2.4. Discussion

This is one of the comprehensive research platforms for detecting AIVs among domestic poultry in Bangladesh. Surveillance findings from 2007-2016 suggest AI A viruses, including HPAI and LPAI subtypes, circulate year-round in domestic waterfowl, commercial chicken, backyard chicken and the environments of LBMs in Bangladesh. LBM is the main place for retail live birds in Bangladesh (Dolberg, 2009). Poultry raisers and local vendors sell chickens, quail, and pigeons alongside waterfowl such as ducks and geese at LBMs. The co-existence of multiple species of avian hosts at households and at LBMs may promote a suitable environment for sustaining, perpetuating and transmitting AI viruses among the poultry population in Bangladesh. Surveillance on AI circulation in LBMs from several countries suggests LBMs are a potential source for AI viruses transmission (Nguyen et al., 2005; Amonsin et al., 2008; Indriani et al., 2010; Negovetich et al., 2011; Wan et al., 2011; Leung et al., 2012).

In southeast Asia, seasonal peaks of HPAI occurred during winter months (Park and Glass, 2007). Our surveillance identified AI virus circulation year-round in both semi-urban and urban LBMs. Avian influenza viruses were detected more in the winter season compared to the summer and monsoon seasons. The role of ducks in the epidemiology of AI is important as they carry and shed influenza A viruses without showing clinical signs. We detected AI A/H5 more in ducks that were apparently healthy than in geese during sample collection. Epidemiological studies suggest that asymptomatic carrier ducks could maintain, perpetuate and transmit HPAI viruses to other susceptible avian hosts (Hulse-Post et al., 2005; Kim et al., 2009). Domestic ducks are reared together with other poultry species, including chickens. Ducks raised in backyard farms appeared to be a risk factor for spreading AI in the environment and to backyard chickens (Gilbert et al., 2006; Henning et al., 2010). We identified AI virus A/H5 more often in backyard ducks than in ducks from small-scale and commercial farms. Backyard ducks scavenge in free range areas including rice fields, ponds, wetlands, canals and rivers. In Bangladesh, 50% of chicken are reared in backyard farms where ducks are allowed to live together during grazing and resting (GoB, 2011). Traditional backyard poultry rearing practices could then promote AI transmission from ducks to backyard chickens.

This surveillance detected AI viruses in environmental samples collected from the rural and urban markets throughout the year with increased identification of H5 subtypes during winter months. The environments of urban LBM were more contaminated with AI viruses than in rural or peri-urban markets. As urban LBM sits everyday without a close day, this situation promotes continuous circulation and maintenance of AI viruses within the poultry population sold at LBM. On the contrary, peri-urban or rural LBM sit either once or twice per week that disrupt continuous circulation of AI viruses. These findings indicate that poultry sold at LBM were infected with AI viruses and shed viruses in the environment of LBM. During 2011 and 2012, there was high level of circulation of AI viruses in environmental specimens. This sudden influx of AI circulation was due to the introduction of new clade 2.3.2.1 of H5N1 which might have substituted the previously circulating clade 2.2 of H5N1 virus. Studies from many countries including Bangladesh reported AI viruses including HPAI H5N1 in environmental specimens collected from LBM (Wang et al., 2006; Nguyen et al., 2005; Indriani et al., 2010; Wan et al., 2011; Shi et al., 2013; Biswas et al., 2015). A study from Indonesia identified AI viruses in poultry water, drains, tabletops, cages, tablecloths, utensils, bins and floor of LBM. Environmental sites belonging to slaughter areas were mostly contaminated because the slaughtering generated droplets with high viral loads (Indriani et al., 2010). An epidemiological study from China tested avian and environmental samples from LBM to detect H7N9 virus and they detected H7N9 in environmental samples (Shi et al., 2013).

Chicken are the major poultry species in urban LBM, whereas waterfowl are abundant in peri-urban and rural LBM. This LBM-based AI surveillance detected a higher prevalence of HPAI in domestic waterfowl (duck and geese) than in commercial chicken and backyard chicken. As waterfowl act as a reservoir for AI and they carry infection without showing clinical manifestation (Kim et al., 2009b). On the other hand, chicken are highly susceptible to HPAI and they are the dead end host. LBM usually sells apparently healthy chicken. Thus, market chickens are less likely to carry HPAI than waterfowl. However, the co-existence of waterfowl and chicken at LBM possibly increases the risk of interspecies transmission of AI and increases the probability of genetic reassortment. A study from Bangladesh identified multiple reassortment events among HPAI viruses detected in poultry population (Gerloff et al., 2014).

H9N2 virus is low pathogenicity in chicken and often causes mild infection with moderate mortality (Shen et al., 2014) and reduced production potential (Brown et al., 2006). H9 was the predominant subtype identified in commercial chicken. Our surveillance identified H9 subtype in 2% of commercial chickens that were sampled from LBMs. The prevalence of H9 subtype in other species was relatively low. However, earlier studies from Bangladesh reported 19% prevalence for H9N2 among market chicken (Negovetich et al., 2011) and 18% sero-prevalence in backyard chicken (Alam et al., 2003). In Hong Kong LBMs, H9N2 subtype was identified in poultry and the prevalence was 4.4% (Shortridge, 1999). H9N2 virus was also prevalent in poultry throughout the Middle East and Asia (Liu et al., 2003; Seo and Kim, 2004; Aamir et al., 2007; Lee et al., 2010; Moosakhani et al., 2010). In China, genotypic analysis revealed that the H7N9 viruses that caused the 2013 outbreak in humans in China were novel reassortants. The HA gene of H7N9 viruses originated from AI viruses circulating in ducks in Zhejiang Province, the NA gene was related to AI viruses isolated from wild birds, and the internal genes probably originated from an earlier H9N2 lineage (Gao et al., 2013). So, H9N2 viruses have lots of evolutionary potential to create novel AI strains among poultry population in future.

The surveillance detected multiple haemagglutinin (HA) subtypes in poultry and in environmental samples of LBM including H1N1, H1N3, H2N4, H3N2, H3N6, H3N8, H4N2, H4N6, H5N1, H5N2, H6N1, H6N7, H7N9, H9N2 and H11N3, . This diversity may increase the probability of genetic reassortment between influenza subtypes that enhances the evolution of a future novel pathogenic strain of animal and public health importance. Surveillance from Korea isolated several subtypes including H9N2, H3N2 and H6N1 in poultry sold at LBM (Seo and Kim, 2004). In USA, subtypes H1, H2, H3, H4, H5, H6, H7, H9, H10 and H11 were isolated from gallinaceous birds, waterfowl and environmental specimens from the LBM between 1993 and 2000 (Panigrahy et al., 2002).

Though the vaccination programme against H5N1 has continued since 2012 in commercial chicken in Bangladesh, our surveillance detected both H5 and H9 subtypes in commercial chicken populations. This concurrence situation indicates that commercial poultry were not protected sufficiently against H5N1 with the vaccine and they therefore shed AI viruses through their feces. These findings demand further study to evaluate vaccine efficacy or compliance to the vaccination program in local settings.

This surveillance identified three different clades of H5N1 viruses in poultry between 2007 and 2013. These clades were 2.2.2, 2.3.2.1 and 2.3.4.2. Other virological studies from Bangladesh identified different types of clades in poultry during the same time period. In 2011, clade 2.2, 2.3.2, 2.3.1 and 2.3.2.1 were identified (Islam et al., 2012; Mondal et al., 2013) and these clades were genetically related to the isolates from neighboring countries including India, Bhutan, Myanmar, Nepal, China and Vietnam (Mondal et al., 2013). Findings from our surveillance and other studies suggest that H5N1 virus of clade 2.3.2.1 has been the predominant strain since its introduction to Bangladesh in 2011.

Haemagglutination inhibition (HI) testing revealed that the identified H9N2 viruses were antigenically identical to an existing WHO candidate vaccine virus: A/Bangladesh/0994/2011. The HA of the H9N2 viruses continues to evolve in the G1 lineages. Evidence of continuous circulation of H9N2 viruses in poultry sold at LBMs suggests that humans are highly likely to get H9N2 infection from LBMs. Though H9N2 causes mild infection in human and poultry, H9N2 viruses should be monitored at animal-human interfaces due to high potentiality of genetic reassortment and production of a future novel strain. A previous study from Bangladesh identified a reassortment event of HPAI (H5N1) virus containing a H9N2-PB1 gene in poultry (Monne et al., 2013).

Since February 2013, a novel reassortment of H7N9 virus has been causing repeated outbreaks in humans, but there was no evidence of outbreak in poultry in China (Chen et al., 2013). In Bangladesh, no outbreak of H7N9 has been identified either in human or poultry populations. However, this surveillance detected very few H7N9 virus in environmental samples collected from LBM that is genetically unrelated to the H7N9 viruses isolated from China. However, a thorough investigation of the Chinese H7N9 strain is warranted in Bangladesh as this H7N9 can be introduced in to the country through different means such as by migratory wild birds or poultry trading.

Our surveillance data, together with government reports on AI outbreaks in poultry demonstrate the repeated introduction of influenza A viruses in the poultry population since 2007. A total of eight human cases were reported from Bangladesh between 2007 and 2018 with one fatal case

(WHO, 2018). Among the eight cases, three were poultry workers (IEDCR, 2012a; IEDCR, 2012b). This coincidence of AI isolation from poultry and humans suggests LBM could play an important role in further dissemination of AI viruses, including H5N1, among poultry and humans in Bangladesh. This is because LBMs collect poultry with potential AI infections from different sources and different geographical locations around the country to be sold and slaughtered.

2.5. Study limitations

This study is subject to multiple limitations. We used convenience sampling to select waterfowl from LBMs because of the small number of waterfowl kept for sale; however the sample size was sufficient to produce meaningful outcomes. In this study, we were only able to sample a small number of waterfowl reared on small-scale and commercial farms and therefore may have underestimated overall AIV circulation in Bangladesh. These surveillance data were only collected in five of the 64 districts in Bangladesh. Over the surveillance period, we changed the sampling number and number of study sites due to funding availability from our donor. Though we performed rRT-PCR on all collected samples to detect AIVs (only subtypes H5, H7 and H9) at the icddr,b lab, only a subset of AI type A positive samples were further tested at the US CDC lab for further characterization of viruses.

2.6. Conclusions

Surveillance findings from 2007-2016 suggest that AI A viruses, including H5, circulate year-round in domestic ducks, geese, commercial chickens and backyard chickens in LBMs in Bangladesh. LBM environments contaminated with H5N1 may act as a potential source of infection in poultry and may increase the risk of avian-to-human transmission. Urban LBMs were at higher risk for environmental contamination with AI than rural or peri-urban LBMs. LBM should be targeted to implement interventions through improved biosecurity and cleaning and disinfection to reduce the transmission of AI viruses. LBM-based surveillance should be continued to develop a better understanding of influenza virus circulation in domestic poultry and to provide a sentinel detection mechanism for novel AI viruses of public health importance.

Chapter-3: Avian Influenza Viruses Seasonality in Domestic Poultry and its Association with Climatic Factors in Bangladesh

3.1. Introduction

Highly pathogenic avian influenza A virus (H5N1) poses a threat to the poultry industry and to human health (OIE, 2018; WHO 2018) across the world. The subtype H5N1 has caused a large number of outbreaks in poultry in Asia, Europe and Africa (OIE, 2018). The first HPAI outbreak in Bangladesh was reported in March 2007 and there have been more than 550 reported HPAI outbreaks to date in 52 out of 64 districts, 90% of which were in commercial poultry farms (OIE, 2018). The people of Bangladesh live close proximity with their poultry which increases the likelihood of the occurrence of HPAI H5N1 in humans (GoB, 2011), although only eight human HPAI H5N1 cases, including one death, have been reported to WHO so far (Brooks et al., 2009; IEDCR, 2012a; IEDCR, 2012b; IEDCR, 2013). The peaks of the HPAI H5N1 outbreak waves in poultry were reported in February- July 2007 and January-April 2008 in Bangladesh (Ahmed et al., 2010).

It has been reported that influenza seasonality is closely related to virus survival, host immunity and effective contact rate. Each of these three factors can be influenced by series of seasonal stimuli like temperature, humidity and rainfall (Tamerius et al., 2011). HPAI H5N1viruses' circulation was higher in winter than warmer months. HPAI viruses persist in cold water or environment for longer time that may promote the transmission of AIVs (Gilbert et al., 2008). However, an epidemiological study from Bangladesh did not find any significant correlation between climatic factors and HPAI H5N1 outbreaks in poultry (Biswas et al., 2014). As most of the AI outbreaks were reported during winter months, the analysis used by this study for temporal patterns of AI was not clearly understood during the course of a full year.

Based on icddr,b AI surveillance findings along with some published studies (Gilbert et al., 2008; Tamerius et al., 2011), it has been hypothesized that low temperatures influence AIV survival, reduce host immunity and increase the likelihood of host contact rate. Year-round

surveillance data is therefore more informative and powerful than any seasonal outbreak data to understand the temporal patterns of AI circulation in Bangladesh. If climate factors are not associated with AI seasonality, other factors including biosecurity practices and poultry management need to be emphasized. In spite of extensive research works on AI epidemiology, the seasonal drivers of AI occurrence in domestic poultry in Bangladesh are not clearly understood. Therefore, a study was conducted to investigate AI at time and space and incorporating climatic parameters in different poultry species (waterfowl, commercial and backyard chicken). The study used a big data set produced through the sentinel surveillance programme during 2007-2016 and had an ultimate aim of developing intervention strategies for preventing and controlling AI in poultry and humans in Bangladesh.

3.2. Materials and Methods

3.2.1. Study Sites and Type of Surveillance

Live Bird Markets in different districts of Bangladesh were chosen for active AI surveillance between October 2007 and December 2016. The selection criteria for the districts and the LBMs are described in Chapter-2. The distribution of LBMs by year and district is also presented in Chapter-2.

3.2.2. Sampling and Climate Data Collection

The operation of active AI surveillance as well as our sampling and data collection methodologies is thoroughly described in Chapter-2. Monthly climate data were extracted from the database provided by Bangladesh Meteorological Department for the period between 2007 and 2016 (BMD, 2017). Bangladesh Meteorological Department has several regional stations for data recording. In this study, we used Dhaka station data as Dhaka is the centre of the country. Averaged maximum and minimum temperatures and humidity data by month were used for the present study. For precipitation data, monthly cumulative rainfall data were considered.

3.2.4. Laboratory evaluation

Swab samples (cloacal and oropharyngeal swabs and environmental samples) were collected as per the protocol described in Chapter-2. RNA extracts were initially evaluated by screening one step real time RT-PCR targeted matrix gene. The positive samples were then evaluated further by gene specific (H5, H7 and H9) one step real time RT-PCR. RNA extraction and rRT-PCR testing protocols are described in Chapter-2.

3.2.5. Statistical Analyses

Periodic cycles or seasonal patterns in the AI time series were estimated using Windowed Fourier analysis or harmonic analysis as previously described (Rogers et al 2002). Fourier analysis was used to sum up the 12-monthly, 6-monthly, and 3-monthly harmonic to create a "Periodic Annual Function" (PAF). The PAF has the seasonal signature of original data on AI, where year-to-year variations (trends and anomalies) were removed, but seasonal variation within the year was preserved. "Epipoi freeware" was used for these analysis and visual representations of the time series (Alonso et al., 2012).

To determine the role of climate on AI seasonality, a pair wise Spearman Correlation Coefficient was estimated between standardized AI time series and climate indicators. The correlation coefficients were expressed as values between -1 and +1. A coefficient of +1 indicates a perfect positive correlation and a coefficient of -1 indicates a perfect negative correlation.

3.3. Results

3.3.1. Detection of Avian Influenza Viruses in Different Domestic Poultry in LBMs

Monthly detection of AIVs in different poultry species between 2007 and 2016 is given in detail in Chapter-2. In waterfowl, AIVs were detected frequently in 2011-2013 and then again in 2016. Contrarily, AIV detection remained constant with low prevalence in the other years. In commercial chicken, a higher proportion of AIVs was determined in 2015 and 2016 compared

with any other years. In backyard poultry AIVs were detected sporadically, however the highest prevalence was estimated in 2014 (particularly in February). In waterfowl, AIVs were detected in winter (December and January) (Figure 3.1). In commercial chicken and backyard chicken, no clear seasonal pattern of AIV detection was determined (Figures 3.2 and 3.3).

3.3.2. Detection of Avian Influenza Viruses in the Environment of LBMs

Monthly detection of AIVs in environmental specimens between 2007 and 2016 is given in detail in Chapter-2. Detection of AIVs was more common in the samples obtained during 2009-2011. In 2014 and 2015, H9 detection was higher than that of H5. Two periodic annual signals of AIVs were pronounced (January and February and then July) (Figure 3.4).

3.3.3. Role of Climate Factors on the Seasonality of Avian Influenza Viruses in Poultry and Environment of LBMs

In waterfowl, AIV circulation was negatively correlated with monthly average temperature (r= -0.31), humidity (r= -0.04), precipitation (r= -0.15) and wind speed (r= -0.34). However, the virus's relationship with humidity and precipitation variables was not statistically significant (Figure 3.5). In commercial chicken, AIV circulation was positively correlated with humidity (r= -0.08) and precipitation (r= -0.02), but negatively correlated with temperature (r= 0.11) and wind speed (r= -0.14). However, the relationship was not statistically significant (Figure 3.6). In backyard chickens, AIV circulation was negatively correlated with all climate variables except wind speed ($p \leq 0.05$) (Figure 3.7). In environmental samples collected from poultry stalls of LBMs, AIV circulation was negatively correlated with temperature (r= -0.16) and humidity (r= -0.04). However, the relationship for environmental sample was not statistically significant (Figure 3.8).

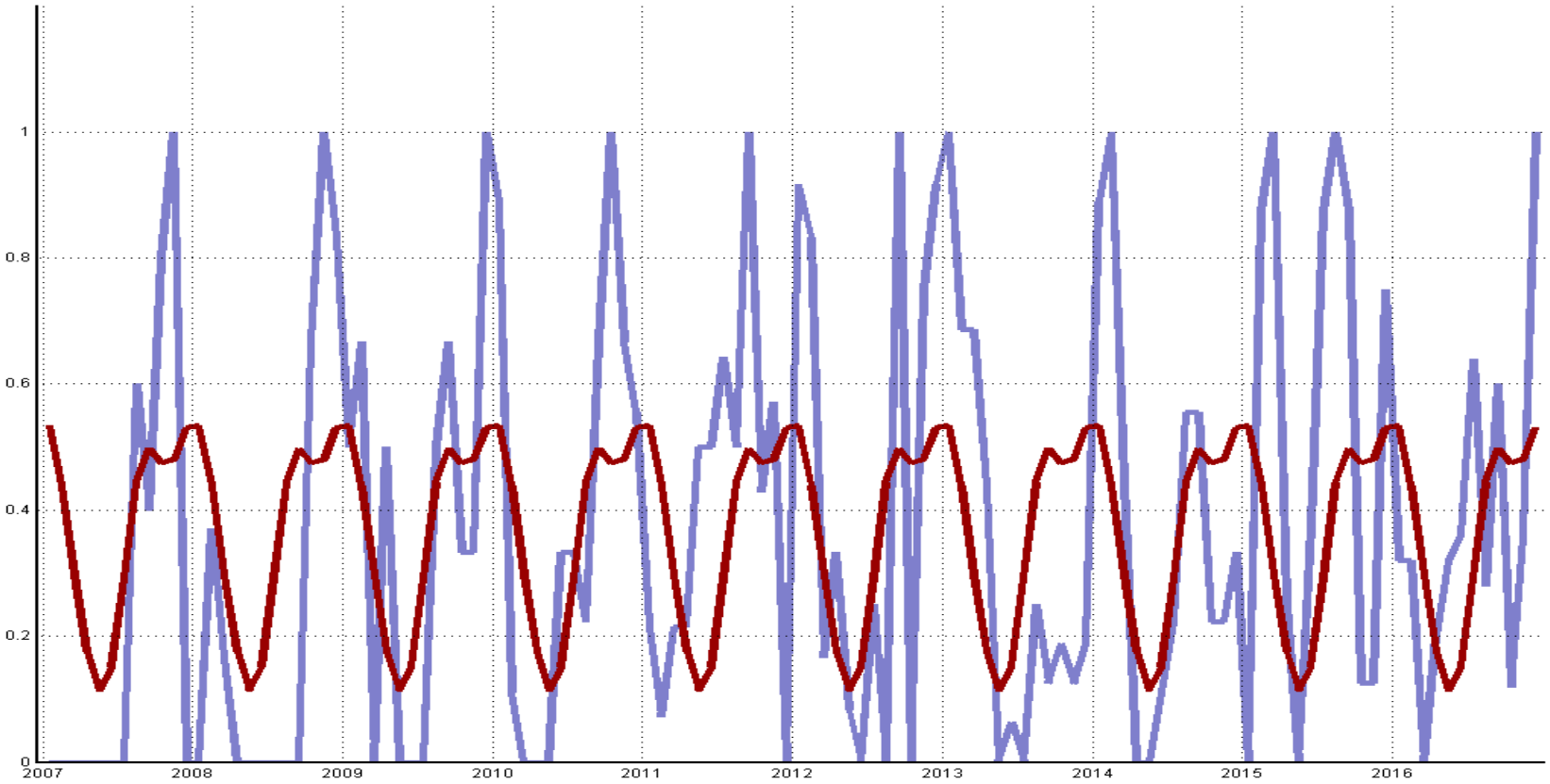


Figure 3.1. Time series of monthly detection of avian influenza viruses standardized from zero to one per year among domestic waterfowl in Bangladesh and seasonal model based on Fourier decomposition from 2007 to 2016. The time series in light blue represents raw data, whereas the overlapped red curve represents the model trend and seasonality. The red line shows the upper limit of 95% confidence interval of the periodic annual function obtained by the sum of the three first harmonics, indicating single peak per year.

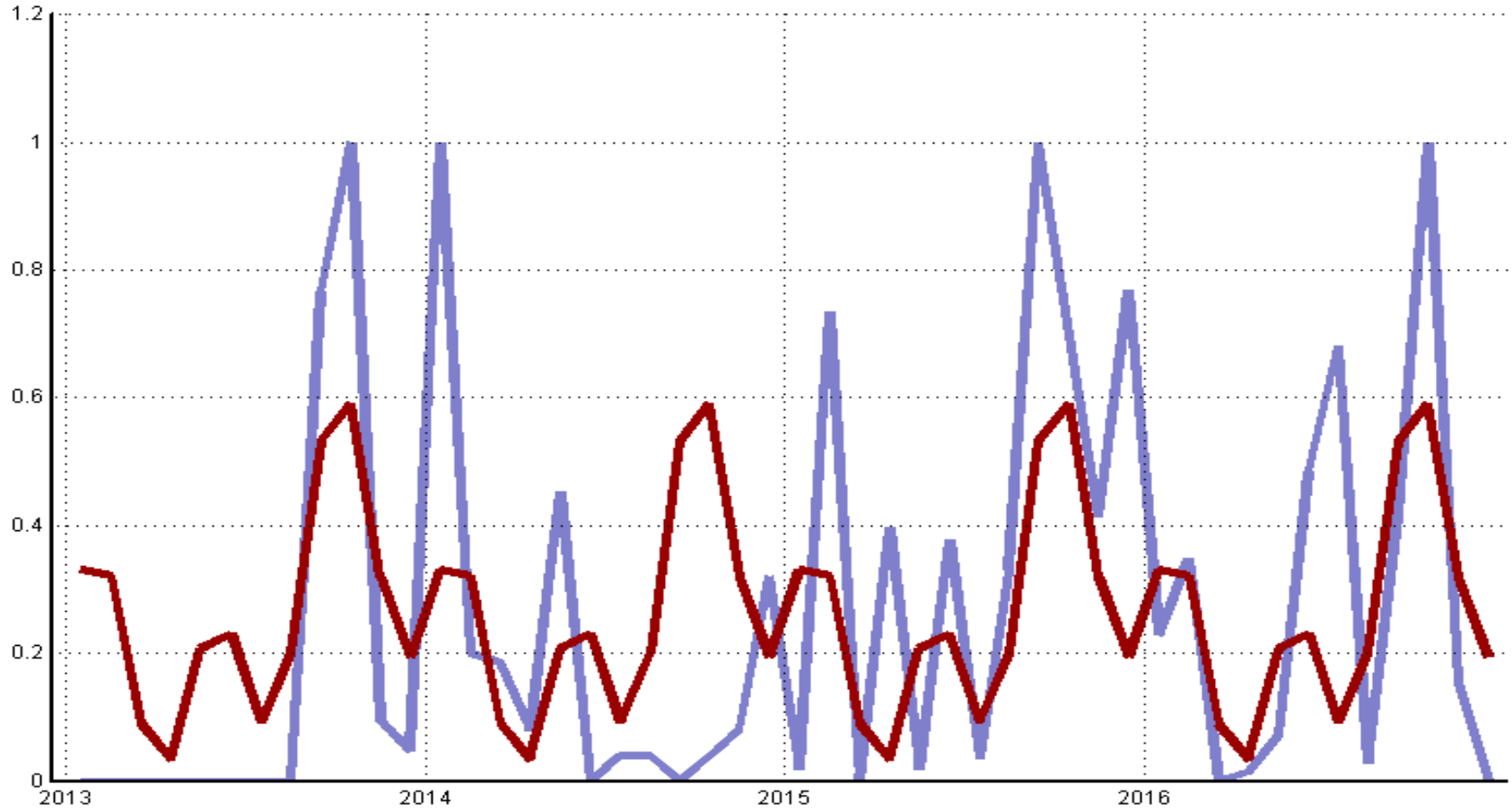


Figure 3.2. Time series of monthly detection of avian influenza viruses standardized from zero to one per year among commercial chicken in Bangladesh and seasonal model based on Fourier decomposition from 2013 to 2016. The time series in light blue represents raw data, whereas the overlapped red curve represents the model trend and seasonality. The red line shows the upper limit of 95% confidence interval of the periodic annual function obtained by the sum of the three first harmonics, indicating three peaks per year.

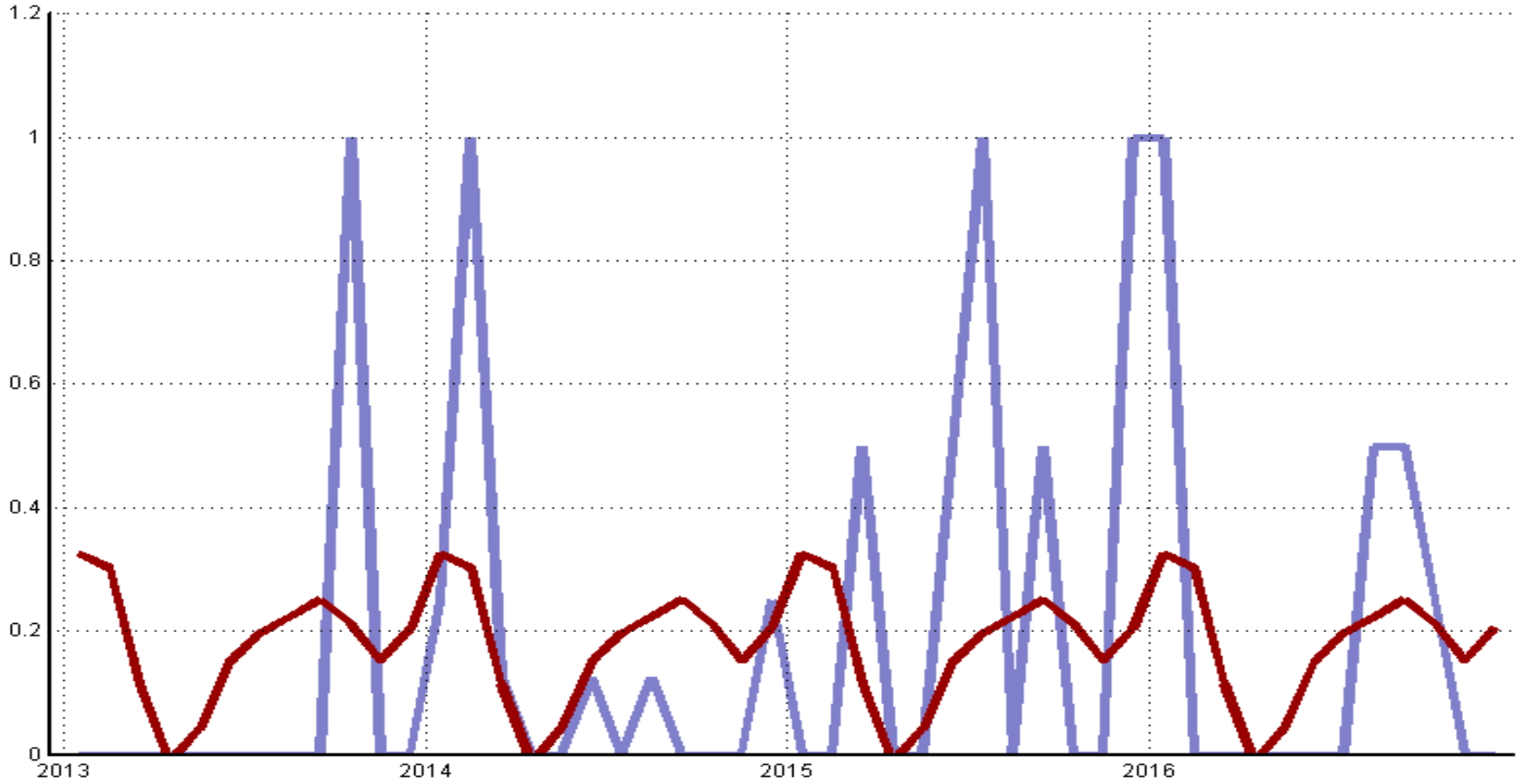


Figure 3.3. Time series of monthly detection of avian influenza viruses standardized from zero to one per year among backyard chicken in Bangladesh and seasonal model based on Fourier decomposition from 2013 to 2016. The time series in light blue represents raw data, whereas the overlapped red curve represents the model trend and seasonality. The red line shows the upper limit of 95% confidence interval of the periodic annual function obtained by the sum of the three first harmonics, indicating two peaks per year.

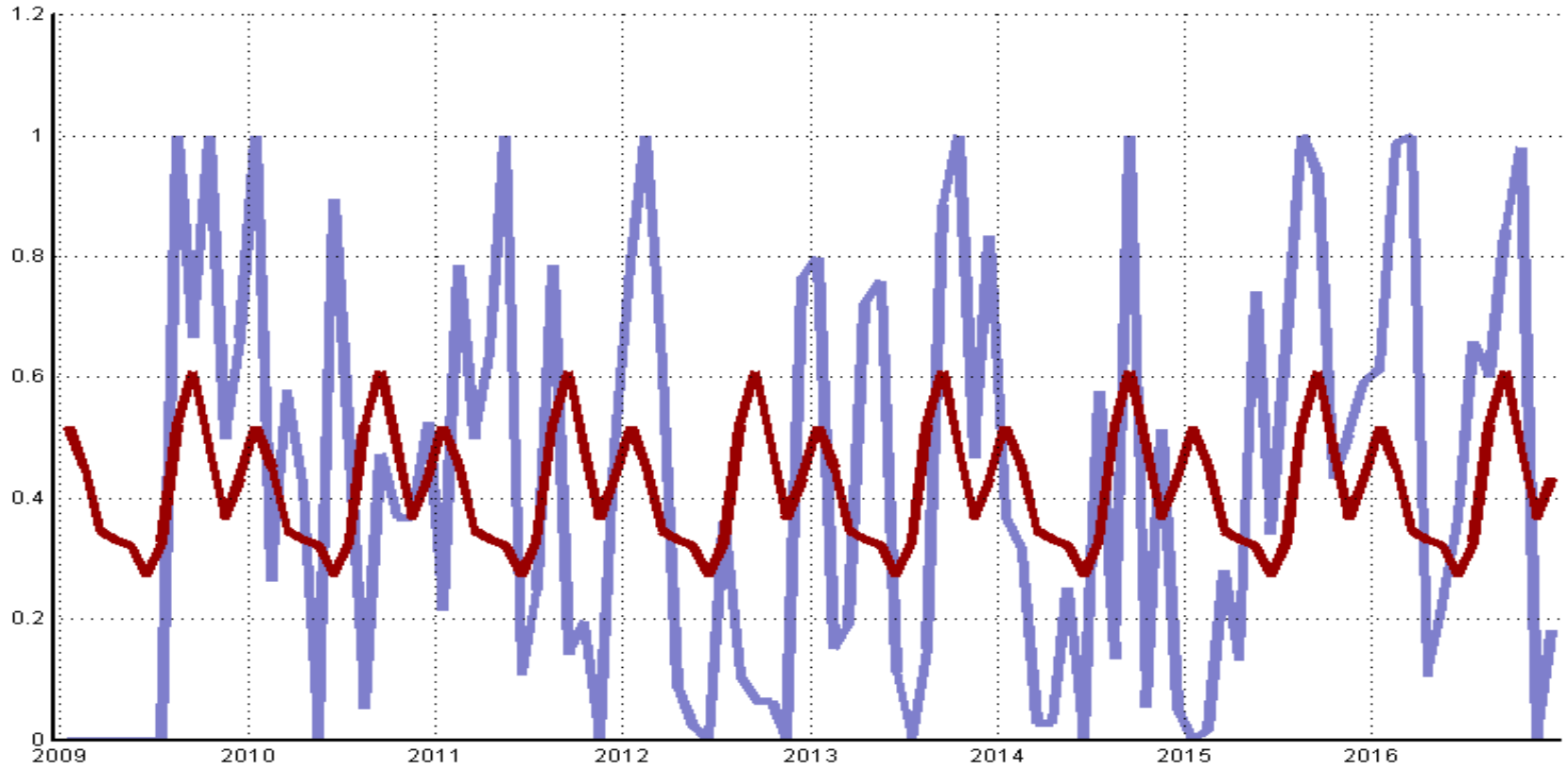
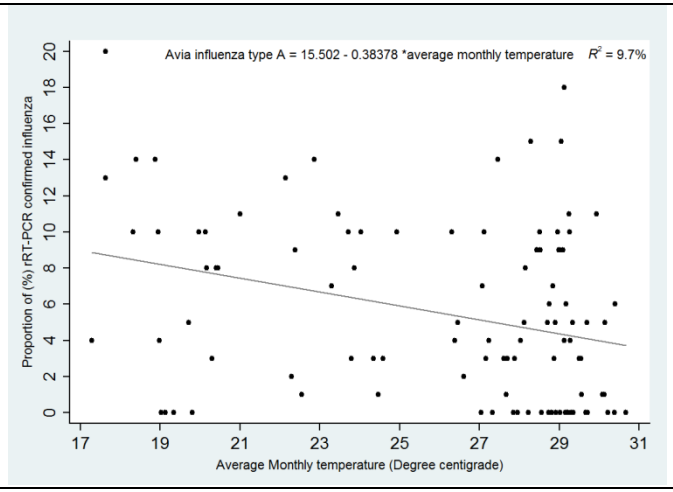


Figure 3.4. Time series of monthly detection of avian influenza viruses standardized from zero to one per year in environmental specimens in Bangladesh and seasonal model based on Fourier decomposition from 2009 to 2016. The time series in light blue represents raw data, whereas the overlapped red curve represents the model trend and seasonality. The red line shows the upper limit of 95% confidence interval of the periodic annual function obtained by the sum of the three first harmonics, indicating two peaks per year.



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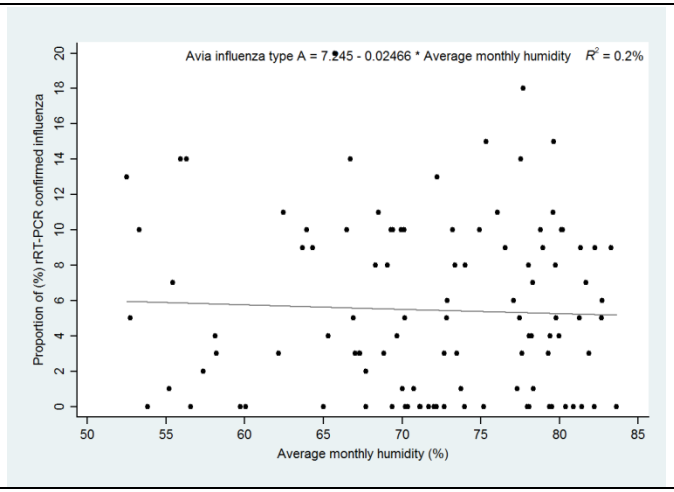

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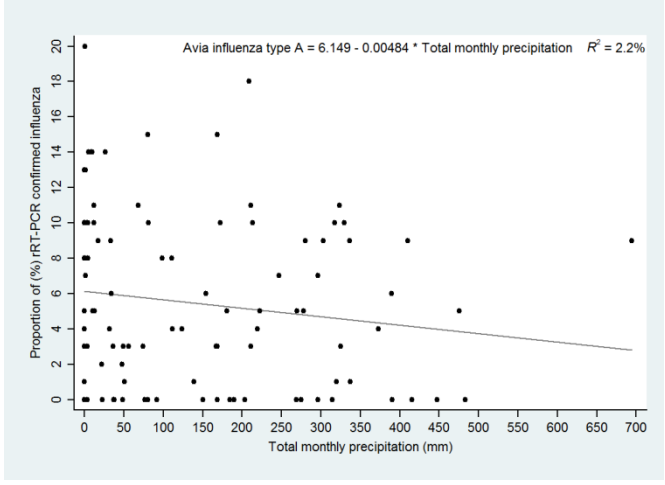

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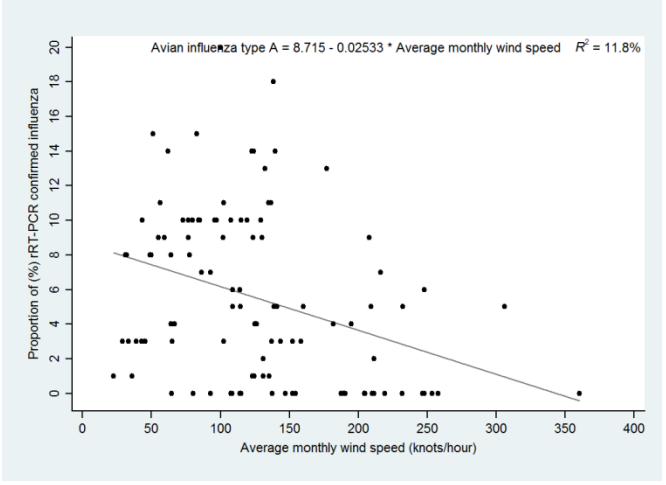
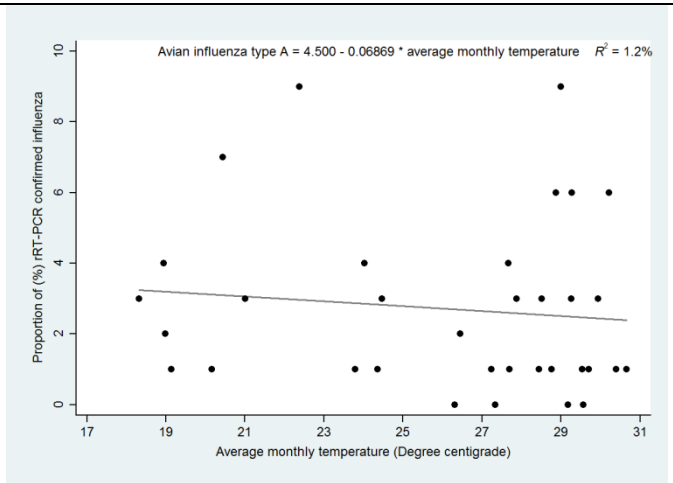


(d)

Figure 3.5. Relationship between monthly detection of avian influenza viruses for waterfowl and climate; (a) avian influenza versus average monthly temperature, (b) avian influenza versus average monthly humidity, (c) avian influenza versus total monthly precipitation and (d) avian influenza versus average monthly wind speed



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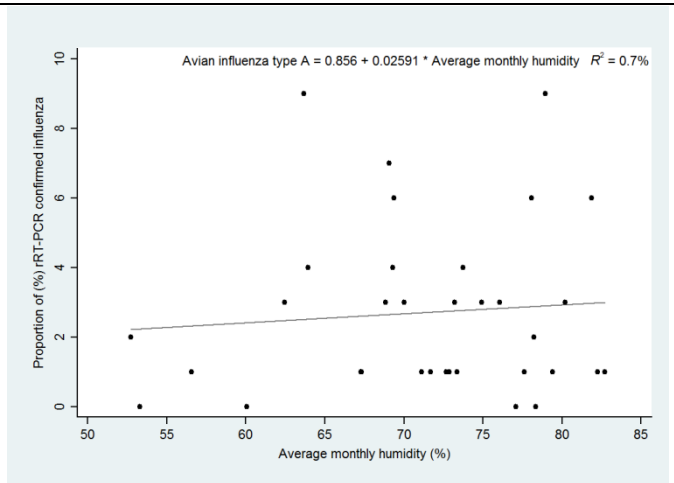

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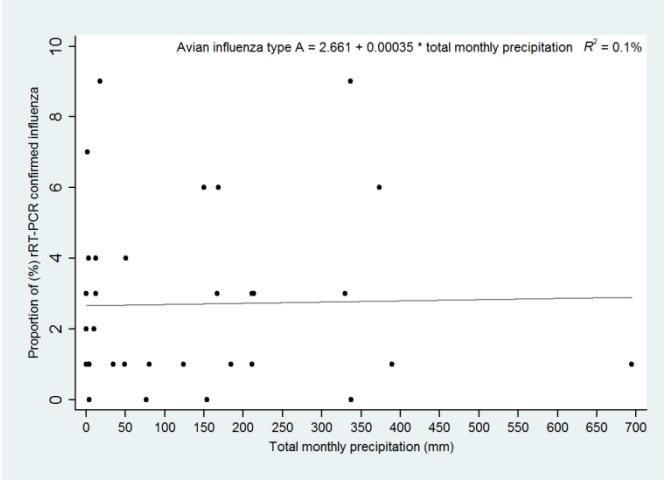

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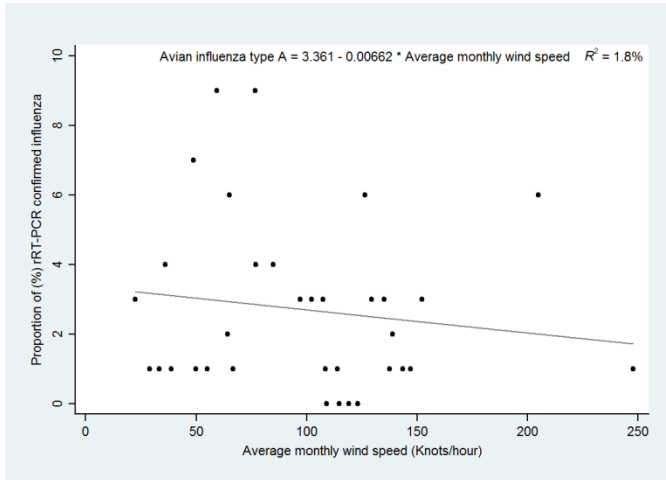
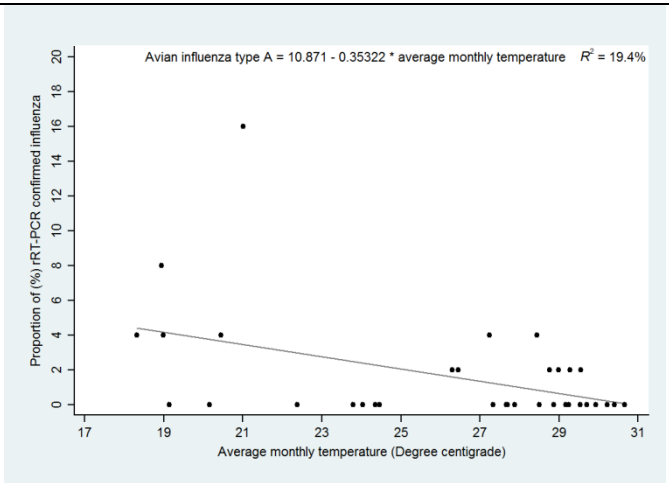


(d)

Figure 3.6. Relationship between monthly detection of avian influenza viruses for commercial chicken and climate; (a) avian influenza versus average monthly temperature, (b) avian influenza versus average monthly humidity, (c) avian influenza versus total monthly precipitation and (d) avian influenza versus average monthly wind speed



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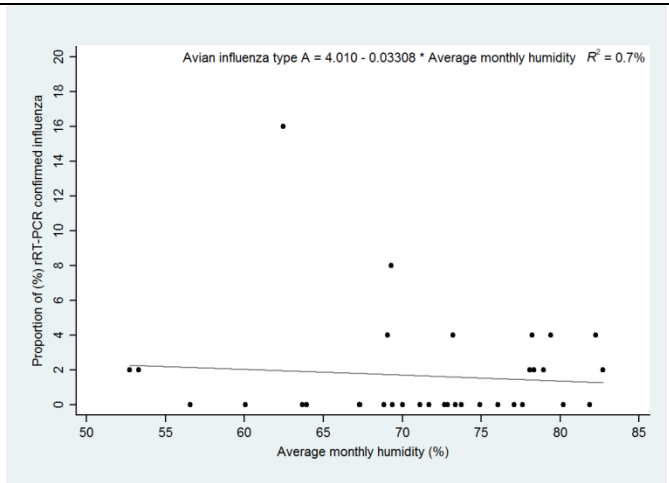

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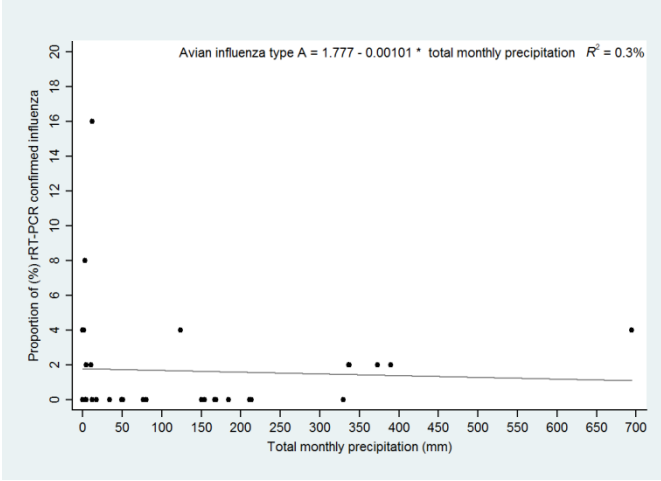

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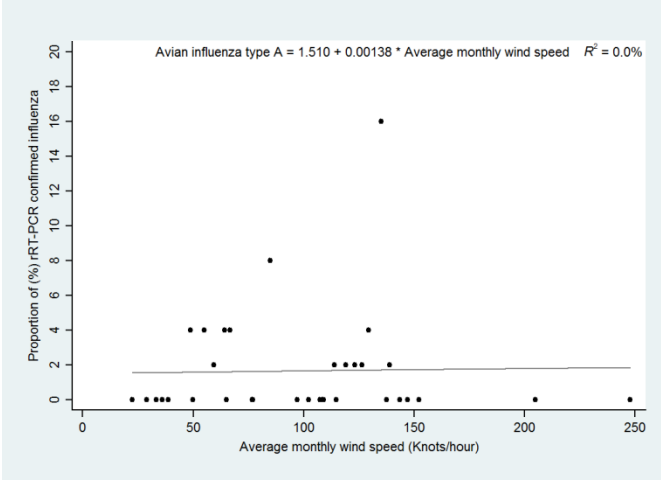

(d)

Figure 3.7. Relationship between monthly detection of avian influenza viruses for backyard chicken and climate; (a) avian influenza versus average monthly temperature, (b) avian influenza versus average monthly humidity, (c) avian influenza versus total monthly precipitation and (d) avian influenza versus average monthly wind speed

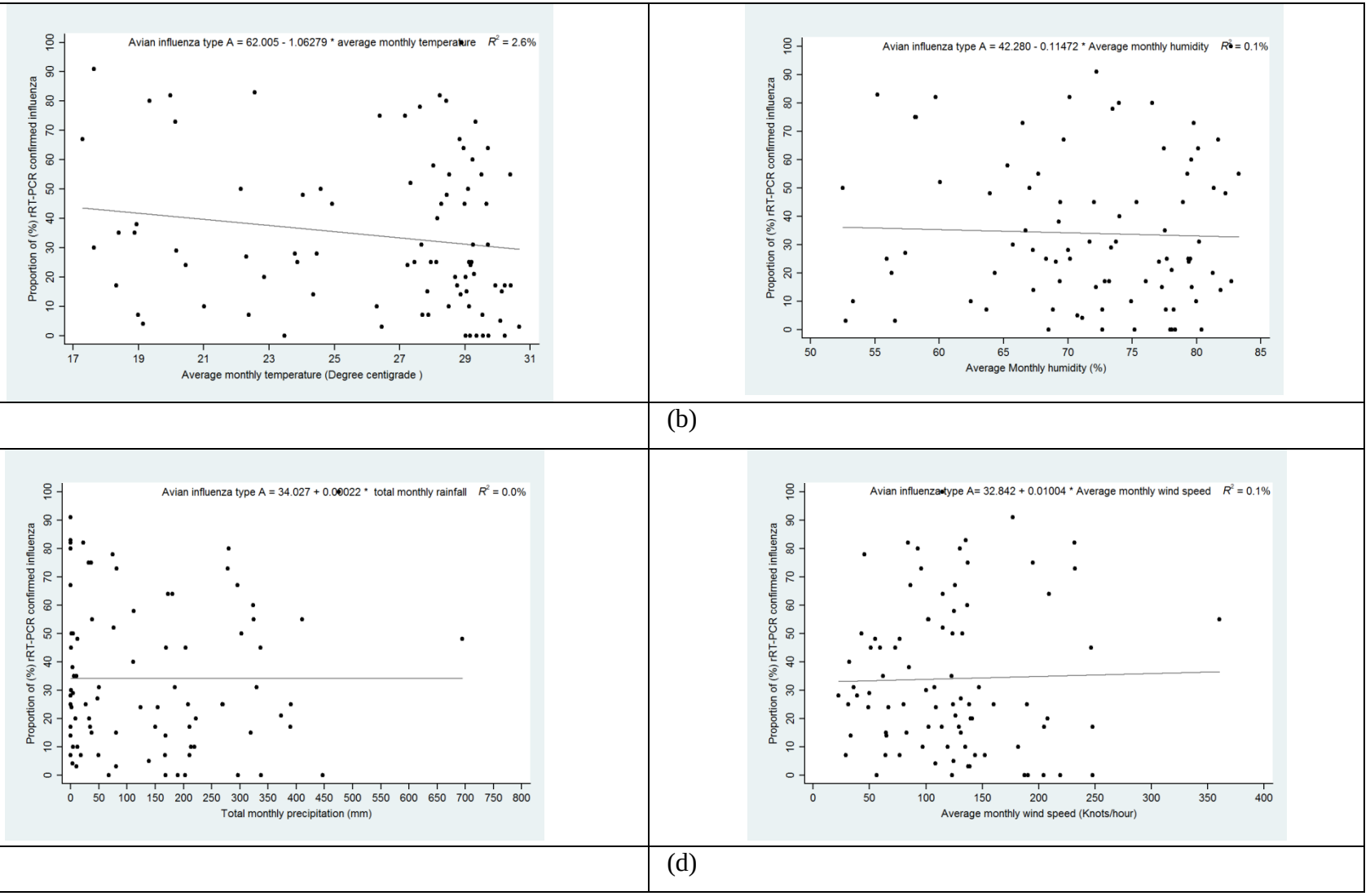

Figure 3.8. Relationship between monthly detection of avian influenza viruses' detection for environmental specimen and climate; (a) avian influenza versus average monthly temperature, (b) avian influenza versus average monthly humidity, (c) avian influenza versus total monthly precipitation and (d) avian influenza versus average monthly wind speed

Table 3.1. Relationship between climate variables and timing of avian influenza viruses circulation

Variables	Waterfowl		Commercial chicken		Backyard chicken		Environmental specimens	
	Correlation coefficient (r)	*P*	Correlation coefficient (r)	*p*	Correlation coefficient (r)	*p*	Correlation coefficient (r)	*P*
Monthly average temperature (^{o}C)	-0.31	0.001	-0.11	0.54	-0.44	0.009	-0.16	0.141
Monthly average humidity (%)	-0.04	0.673	0.08	0.64	-0.08	0.640	-0.04	0.746
Monthly total precipitation (millimeter)	-0.15	0.134	0.02	0.90	-0.05	0.775	0.001	0.991
Wind speed (Knots per hour)	-0.34	0.001	-0.14	0.46	0.02	0.902	0.02	0.827

3.4. Discussion

Bangladesh is sub-tropical country having six different seasons in a year: summer (mid April to mid June), monsoon (mid June to mid August), autumn (mid August to mid October), late autumn (mid October to mid December), winter (mid December to mid February) and spring (mid February to mid April). However, winter, summer and monsoon are the prominent seasons in Bangladesh. The average minimum temperature for winter ranges from 7-13° C and the maximum average temperature ranges 24-31°C. The average maximum temperature in the summer months is around 37°C. The average annual rainfall varies from 1429 to 4338 millimeters (BBS, 2015b) .

Globally, HPAI H5N1 outbreaks show a clear seasonal pattern in poultry, humans and wild birds. Most human cases (50%) are reported during January to March (Durand et al., 2015). In poultry, most H5N1 outbreaks are detected in winter and early spring (i.e., October to March) (Auewarakul, 2008, Si et al., 2009; ElMasry et al., 2017; OIE, 2017a). A European surveillance detected peak prevalence for HPAI H5N1 in wild migratory birds in September, October and January (Munster et al., 2007). In Southeast Asia, most HPAI H5N1 outbreaks were detected in colder months (December-March) during 1997-2006 (Park and Glass, 2007). In China, most H5N1 outbreaks were reported in poultry during winter and spring (Li et al., 2015). In our present study, we found that the timing of AI activity varied by poultry species. Circulation of AIVs in waterfowl occurred quite regularly in winter compared with other poultry species. This pattern could be due to the cooler environmental temperature that could promote circulation of AIVs during the winter season. In contrast, the seasonality pattern was irregular in chicken and varied by year. Avian influenza viruses circulation was maintained at certain level in commercial chicken throughout the year that indicate AIVs in commercial chickens appear to be endemic in nature. Though most of the AI outbreaks were reported previously in commercial and backyard chicken during winter (OIE, 2013), the seasonal irregularities for commercial and backyard chicken observed in this study is not well understood.

Spatio-temporal patterns of AI was studied previously using AI outbreak data in different poultry sectors in Bangladesh (Ahmed et al., 2011). The peaks of the HPAI H5N1 outbreak waves were

reported to be occurred in February- July in 2007 and January-April in 2008 (Ahmed et al., 2010). Activities of migratory birds during winter (November to March) are more common in Bangladesh which may have a potential role in the introduction and spread of AI in poultry sectors in this country from Central Asia and East Asia. The connection of AI occurrence with migration of wild birds during winter and spring months was reported by other global studies (Feare, 2010; Lycett et al., 2016). However, an earlier study from Bangladesh did not find any clear seasonality for AI detection (Turner et al., 2017).

Ducks are considered as natural reservoir for AI and they have seasonal infection. A study conducted in USA reported that the onset of AI infection in ducks occurred at nearly the same time every year, particularly in late July or early August (Halvorson et al., 1985). In Vietnam, most H5N1 outbreaks during 2006-2007 were reported during the rainy season. The study suggested extensive rain resulted in temporal flooding that could promote re-emergence of HPAI H5N1 (Henning and Pfeiffer, 2009).

In Europe, the occurrence of HPAI H5N1 in wild birds was highly correlated with the increased normalized difference vegetation index (NDVI) in December; intermediate NDVI in March; increased minimum temperatures in January; and reduced precipitation in January (Si et al., 2010).

A case-control study from China found that 128 HPAI H5N1 outbreaks in poultry and wild birds as well as 21 human cases were significantly associated with minimal distance to the nearest national highway, annual precipitation and the interaction between minimal distance to the nearest lake and wetland. The risk of HPAI H5N1 outbreaks was increased, when the precipitation in a region was decreased (Fang et al., 2008).

Lower environmental temperature was identified as the main factor for seasonality of AI. Avian influenza viruses persist in cold water for a long time and this may be associated with a higher chance of AIVs transmission (Stallknecht et al., 1990). A study suggested that lower ambient temperature may decrease immunity of poultry and make poultry more susceptible to H5N1 virus (Durand et al., 2015). However, HPAI H5N1 persists endemically in Indonesia where high

temperatures and high humidity constantly exist. Germany reported HPAI H5N1 virus twice, once in the middle of winter 2006, and once in mid-summer 2007 (Gilbert et al., 2008). The risk of AI transmission increases during winter in the Northern Hemisphere (OIE, 2017a).

Monthly average temperature was the only significant climate factor that can predict AI activity and seasonality in poultry. The inconsistent association between AI activity and other climate factors (humidity, precipitation and wind speed) found in this study suggests that climate factors might not be so significant a predictor for AI seasonality among poultry in Bangladesh. The role of climate factors on AI seasonality is difficult to explain by the findings of this study. Therefore, other factors such as management and biosecurity practices should be more of a focus for controlling AI transmission among poultry populations.

3.5. Study limitations

Though Bangladesh has several weather stations to record local climate data, we did not find area-specific climate data for all study areas. In this study we generalized the Dhaka city climate data to other surveillance areas. The climate data used for this study may not exactly be representative for all surveillance areas. Therefore, the findings of this study should be interpreted cautiously. This uncertainty warrants a future study with area-specific climate data.

3.6. Conclusion

The timing of AI activity varies by poultry species. Circulation of AIV was seasonal in waterfowl (mostly in winter season: November to March). However, year-round circulation of AIVs in commercial chicken sold at LBMs provides evidence that AI is endemic among commercial chicken in Bangladesh. Domestic waterfowl can play an important role in the transmission of AIVs to other poultry species and humans during winter. The decreasing monthly average temperature is the only significant climate factor that might be associated with increasing AI activity in poultry. Year-round surveillance should be continued and enhanced to detect AIVs including novel subtypes in domestic waterfowl, commercial and backyard chicken.

Chapter-4: Association between Biosecurity Practices and Environmental Contamination with Avian Influenza Viruses in Live Bird Markets, Bangladesh

4.1. Introduction

Highly Pathogenic Avian Influenza (HPAI) H5N1 causes severe infection in poultry and humans (OIE, 2018; WHO, 2018). There have been more than 550 reported HPAI H5N1 outbreaks in poultry sector in Bangladesh since the first reported case in 2007, 90% of which were reported from commercial poultry farms (OIE, 2018). Eight human H5N1 cases, including one death, have been reported from Bangladesh and three of them were poultry market workers (IEDCR, 2012a; IEDCR, 2012b; IEDCR, 2013). A previous study reported that 2% of poultry workers had evidence of antibodies against H5N1 (Nasreen et al., 2015). Therefore, poultry workers are at risk of contracting AI from exposure to infected poultry sold at LBMs.

Many Asian countries including Vietnam, Thailand, Indonesia, Hong Kong, China and Cambodia reported human cases of H5N1 infections with a history of poultry exposure at Live LBMs, suggesting that LBMs could be a potential source for H5N1 infection among poultry and humans (Webster, 2004; Wan et al., 2011,). Bangladesh has more LBMs in urban areas, where multiple poultry species from backyard and commercial production systems are kept together for sale, than in rural areas. Studies have detected AI in market poultry and in the environment of LBMs in Bangladesh (ICDDRB, 2013; Biswas et al., 2015).

Some countries applied interventions to reduce AI spread at LBMs including permanent closure, banning overnight poultry storage, and weekly rest days followed by disinfection of surfaces to reduce environmental contamination of AI (Bulaga et al., 2003; Kung et al., 2003; Lau et al., 2007; Trock et al., 2008; Indriani et al., 2010; Murhekar et al., 2013; Fournié et al., 2013). A previous study from Bangladesh evaluated market-level interventions including cleaning and disinfecting to reduce environmental contamination in LBMs, but they did not find a significant difference in the reduction of AI between intervention and non-intervention markets (Biswas et

al., 2015). Although poultry farm-level and market-level risk factors for HPAI, including biosecurity practices, were studied earlier (Biswas et al., 2009a; Biswas et al., 2009b; Biswas et al., 2011; Ahmed et al., 2012; Biswas et al., 2015), shop-level biosecurity practices in relation to environmental contamination with AI have not been comprehensively studied across the country; though Sayeed et al. conducted a small study in Chittagong (Sayeed et al., 2017). We therefore hypothesized that shop-level bio-security practices are more important than market-level biosecurity practices to reduce environmental contamination of AI. Poultry shops with poor biosecurity practices can be more contaminated with AI. A better understanding about the whole biosecurity system of poultry shops with regards to its role in environmental contamination with AI is necessary to identify poor biosecurity practices to target for further improvement. Our study aimed to estimate the shop-level prevalence of environmental contamination with AI and to identify the association between biosecurity practices and environmental contamination with AI at the shop level.

4.2. Materials and Methods

We conducted a cross-sectional study in ten metropolitan cities in Bangladesh. We choose these cities because of the presence of LBMs that provide a strong interface between poultry and humans.

4.2.1. Selection of LBMs and Poultry Shops

The calculated sample size was 800 poultry shops to detect 1% prevalence of AI with 80% study power at 95% confidence interval. Initially, the field team visited each city to identify all LBMs and to count the number of poultry shops in each LBM. For each city, we prepared a list of LBMs that had at least 10 poultry shops (Appendix VIII). Then, we selected a total of 80 LBMs from the aforementioned list of LBMs from the ten cities using proportionate random sampling. From the list of shops in a LBM, we enrolled ten shops using random number generator in the computer to collect data and environmental specimens. However, if we had 10 shops in a LBM, all of them were recruited.

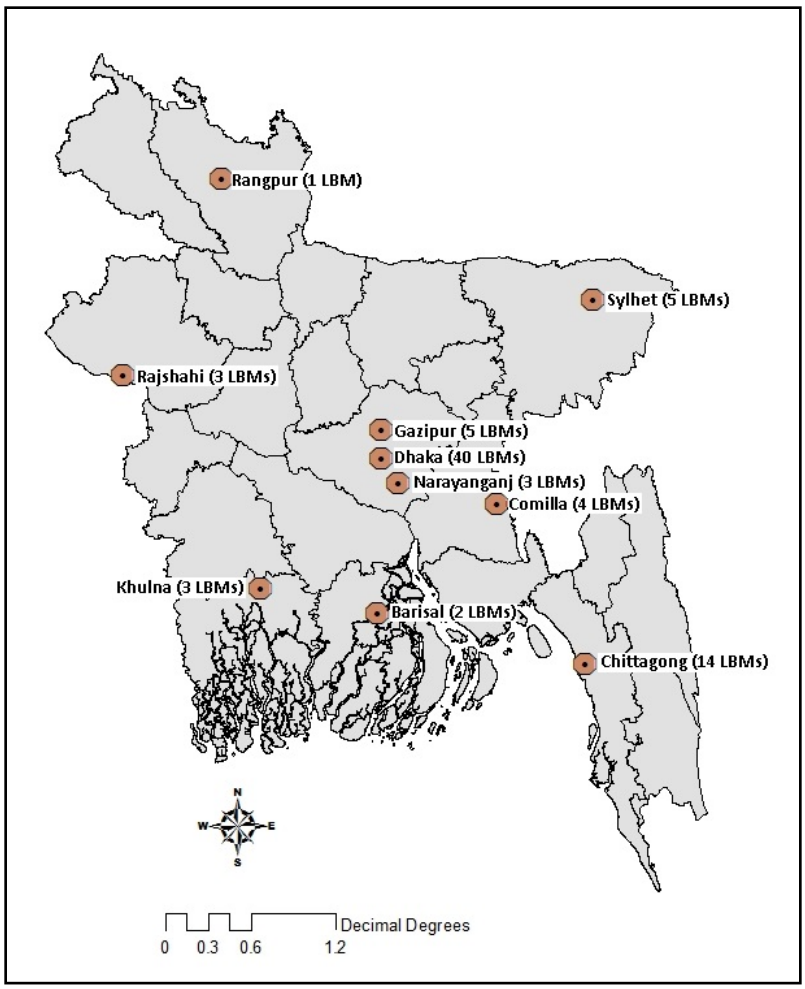

Figure 4.1. Location of 80 selected LBMs in ten metropolitan cities in Bangladesh

4.2.2. Biosecurity Practices

In March 2015, the field team, consisting of 3 members, visited each selected shop to interview either poultry vendors or poultry workers and collected data on shop characteristics, poultry transactions and biosecurity practices using a pretested structured questionnaire. We chose several biosecurity practices in this study considering the plausibility of an association with environmental contamination. The biosecurity practices included cleaning and disinfection practices, overnight poultry storage, presence of a rest day, separation of sick poultry and workers visiting other LBMs (Figure 4.3). In the questionnaire, we defined cleaning as "cleaning with water and/or broom" and we defined disinfection as "cleaning with a disinfectant". We

summarized the role of biosecurity practices and their plausible association with environmental contamination in Appendix IX.

4.2.3. Sample Collection

From each selected shop, we collected swab specimens from different environmental surfaces during interviews. For each shop, we used 8-10 swab sticks to swab multiple surfaces/sources including poultry droppings, cages, feed, drinking water, water used by poultry handlers, slaughtering surfaces, slaughtering byproducts, offal, shop floors, waste bins, and various pertinent utensils like slaughtering knives, to prepare a pooled environmental specimen. A proportion of shops had no slaughter facilities within their premises. From these shops, we collected swabs from other sources including poultry droppings, cages, feed, drinking water and water used by poultry handlers.

4.2.4. Laboratory Testing

An one-step real-time reverse transcription (RT)-PCR detection kit was used for typing and sub typing influenza viruses using fluorescent TaqMan probes as indicator system in icddr,b's laboratory (CDC, 2013). Primers and probes specific for matrix (M) gene were included to detect any of the 16 types of influenza A viruses. To identify H5 subtype in the influenza A virus positive samples, H5 hemagglutinin (HA) gene specific-primers and probes were used as has been described in Chapter 2 (CDC, 2013).

4.2.5. Observation

Based on laboratory testing results, we identified all AI type A/H5 positive shops and an equal number of AI negative shops that were selected using a random number generator from the list of AI type A negative shops for observation. We conducted three hours of short observation in AI positive and negative shops. We determined observation hours for individual selected shops based on their preferred time for cleaning and/or disinfecting. During the observation, we collected information about cleaning and disinfection practices.

4.2.6. Statistical Analyses

We summarized the characteristics of poultry shops including infrastructure and biosecurity measures by descriptive analysis and estimated the prevalence of environmental contamination for avian influenza with 95% confidence interval in shops. We performed bivariable analysis between the variables of biosecurity practices and environmental contamination with avian influenza to calculate prevalence ratio (PR) for crude association. For further analysis, we considered only those exposure variables associated with outcomes with p-value≤0.2. For bivariable and multivariable analysis (where we reported confidence interval and p-value), we always adjusted market level and city level clustering effect together. We constructed a conceptual framework (Figure 4.3 and 4.4) to identify causal associations between variables of interest and to identify confounders as described in conceptual framework (Greenland et al., 1999). Finally we performed multivariable analysis to estimate adjusted prevalence ratio (APR) using generalized linear mixed-effect models with account to cluster effect for city and market levels together and took into account for confounders identified in the conceptual model. We conducted the same statistical analysis plan in market level variables based on conceptual framework. For market level analysis, we adjusted city level clustering effect.

4.3. Results

4.3.1. Characteristics of Poultry Shops and Poultry Transaction Data

The average number of poultry shops for each LBM was 20 (standard deviation: ± 10.5, range: 10-55). No weekly rest days were reported in 74% of poultry shops. Chicken was the major poultry species sold at LBMs (Table 4.1). Poultry shopkeepers accommodated poultry in different confined settings including metallic cages, bamboo baskets and on the floor. Poultry vendors collected poultry from multiple sources (Table 4.3). The average size of a poultry shop was nine square meters and 80% of the poultry shops had uneven floor surfaces, partly made with concrete and mud.

4.3.2. Shop-Level Biosecurity Practices

Most of the shops (91%) had a single poultry species during the day of our visit. We found 6% of shops had waterfowl and 4% kept chicken and ducks together. We found 654 (82%) shops had unsold poultry from the previous day. The cleaning of poultry holding areas was performed on a daily basis in 59% of shops and 23% of shops used disinfectant once per week. Three quarter of shops reported that they often did not follow the recommended weekly rest day. The majority of the poultry shops (85%) slaughtered poultry within the shop premises.

4.3.3. Laboratory Results for Environmental Specimens

Of the 800 sampled shops, environmental specimens from 205 (26%) shops were confirmed for AI A viral RNA. We detected subtype H5 in 31 (4%) shops, H9 in 108 (14%), and both H5 and H9 in 29 (4%). A total of 37 (5%) AI A positive shops remained un-subtypable after icddr,b lab tests (Table 4.2).

Our study identified the AI type A virus in all cities and H5 subtype was identified in seven cities (Figure 4.2). Among the 80 LBMs, we detected RNA for AI type A virus in 74 (93%) and H5 subtype specific RNA in 35 (44%). We considered a market as positive when at least one shop from a market was confirmed for AI.

Table 4.1. Summary statistics for poultry business of investigated poultry shops in March 2015 (n=800)

Type of business				**Shop no. (%)**
Retail				617 (77)
Wholesale				4 (1)
Mixed (both retail and wholesale)				179 (22)
Average size of the poultry shop in square meters, mean (standard deviation)				9 ±7.4
Average trading hours per day, mean (standard deviation)				14.2 ±1.8
Shop wise poultry transaction per day	**Number of shops (%)**	**Mean number of poultry (inter-quartile range)**		
		Stocked/day	**Sold/day**	**Leftover/day**
Only chicken	722 (90)	210 (315)	159 (276)	52 (86)
Only waterfowl	3(1)	130 (139)	108 (125)	22 (14)
Only pigeon	5 (1)	90 (38)	41 (30)	49 (17)
Two poultry species	57 (7)	264 (279)	182 (192)	82 (146)
More than two poultry species	13 (2)	522 (779)	296 (303)	227 (513)

Table 4.2. Shop wise laboratory test results for environmental contamination with avian influenza viruses in ten metropolitan cities

Metropolitan cities	**Number of LBM investigated**	**Total shops tested**	**No. of shops positive for influenza A (%)**	**No. of shops positive for influenza A/H5 (%)**
Dhaka	40	400	116 (29)	46 (12)
Chittagong	14	140	15 (12)	3(2)
Rajshahi	3	30	7 (23)	1 (3)
Sylhet	5	50	25 (50)	2 (4)
Khulna	3	30	3 (10)	0
Barisal	2	20	5 (25)	0
Rangpur	1	10	5 (50)	3(30)
Gazipur	5	50	14 (28)	1 (2)
Comilla	4	40	5 (13)	0
Narayanganj	3	30	10 (33)	4 (13)
Total	**80**	**800**	**205 (26)**	**60(8)**

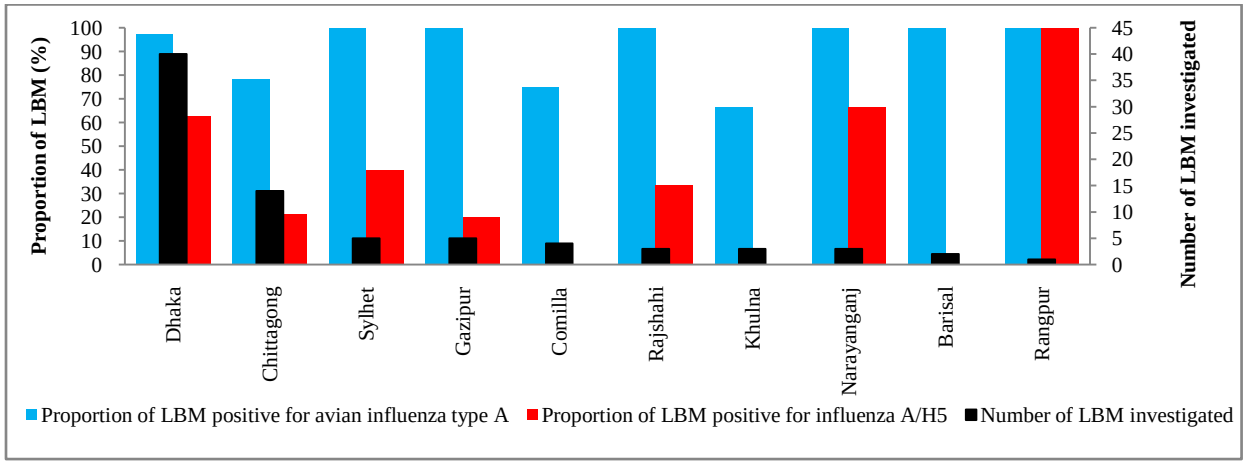

Figure 4.2. Market wise avian influenza viruses detection in ten metropolitan cities

Table 4.3. Shop-level biosecurity practices associated with environmental surfaces contamination by avian influenza viruses (n=800)

Variables	n (%)	Bi-variable analysis			Multivariable analysis		
		PR	95% CI	*p*-value	Adjusted PR	95% CI	*p*-value
Number of poultry species							
Keeping single poultry species	731 (91)	Ref.					
Keeping multiple poultry species	69 (9)	1.3	0.9-1.7	0.130			
Presence of waterfowls							
No	752 (94)	Ref.					
Yes	48 (6)	1.4	0.9-2.2	0.176			
Types of poultry production system							
Commercial poultry	444 (55)	Ref.					
Backyard poultry	53 (7)	1.3	1.0-1.8	0.026			
Mixed (backyard and commercial poultry)	303 (38)	1.4	0.9-2.1	0.097			
Poultry holding areas							
Only wire cage	281 (35)	Ref.					
Only bamboo cage	153 (19)	1.7	0.9-3.2	0.088			
Only floor	24 (3)	2.5	1.1-5.7	0.027			
Mixed	342 (43)	1.5	1.0-2.2	0.080			
Cleaning poultry holding areas							
Not cleaning	26 (3)	Ref			Ref.		
Monthly	68 (9)	0.3	0.2-0.6	<0.001	0.5	0.2-1.0	0.059
Weekly	238 (30)	0.5	0.4-0.8	0.002	0.8	0.5-1.2	0.282
Daily	468 (58)	0.6	0.5-0.7	<0.001	0.9	0.6-1.3	0.472
Disinfecting poultry holding areas							
Not disinfecting	577 (72)	Ref					
Monthly	38 (5)	1.1	0.6-1.7	0.833			
Weekly	185 (23)	0.9	0.7-1.0	0.075			
Slaughtering poultry within shop							
No	115 (14)	Ref.			Ref.		
Yes	685 (86)	1.5	1.1-2.2	0.013	1.6	1.1-2.3	0.018
Number of unsold poultry after the end of business day							
No poultry left	146 (18)	Ref.			Ref.		
Presence of unsold poultry	654(82)	1.9	1.3-2.8	<0.001	1.9	1.3-2.8	0.001
Weekly rest day							
Yes	208 (26)	Ref.			Ref.		
No	592 (74)	1.2	1.1-1.4	<0.001	1.2	1.1-1.4	0.003
Source of poultry							
Poultry farm	49 (6)	Ref.					
Via middlemen	54 (7)	0.9	0.4-2.1	0.804			
Wholesale market	525 (66)	1.0	0.6-1.8	0.857			
Multiple sources	172 (21)	1.0	0.5-1.9	0.900			
Separation of sick poultry from healthy flock							
Yes	357 (45)	Ref.			Ref.		
No	443 (55)	1.2	1.0-1.4	0.048	1.2	1.0-1.4	0.022
Type of shop floor							
Made by tiles/concrete	244 (31)	Ref.					
Dirt/mud	33 (4)	2.4	1.3-4.2	0.003			
Mixed (partial tiles/concrete and mud)	523 (65)	2.6	1.2-3.5	<0.001			

Poultry vendors visited other LBM today							
Yes	136 (17)	Ref.			Ref.		
No	664 (83)	1.2	1.0-1.3	0.055	1.1	0.9-1.3	0.565
Poultry vendors visited other poultry shop							
Yes	506 (63)	Ref.					
No	294 (37)	1.0	0.9-1.2	0.635			
Last two days poultry morbidity							
No	622 (78)	Ref.					
Yes	178 (22)	1.1	0.9-1.4	0.427			
Last two days poultry Mortality							
No	635 (79)	Ref.					
Yes	165 (21)	1.2	0.9-1.4	0.228			

Table 4.4. LBM level-biosecurity practices associated with environmental surfaces contamination by avian influenza viruses (n=80)

Variables	**n (%)**	**Bi-variable analysis**		
		PR	**95% CI**	***p*-value**
Number of shops				
≤20	53 (66)	Ref.		
≥21	27 (34)	1.0	0.9-1.1	0.982
Poultry density				
≤32 poultry per square meter	54 (68)	Ref.		
≥33 poultry per square meter	26 (32)	1.1	0.9-1.2	0.413
Cleaning managed by market committee				
No	28 (35)	Ref.		
Yes	52 (65)	1.0	0.9-1.1	0.891
Disinfection managed by market committee				
No	53 (66)	Ref.		
Yes	27 (34)	0.9	0.8-1.2	0.986
Presence of drain for liquid waste disposal				
Present	60 (75)	Ref.		
Absent	20 (25)	1.0	0.9-1.0	0.233
Presence of central slaughtering facility				
Yes	17 (21)	Ref.		
No	63 (79)	1.0	0.8-1.1	0.772

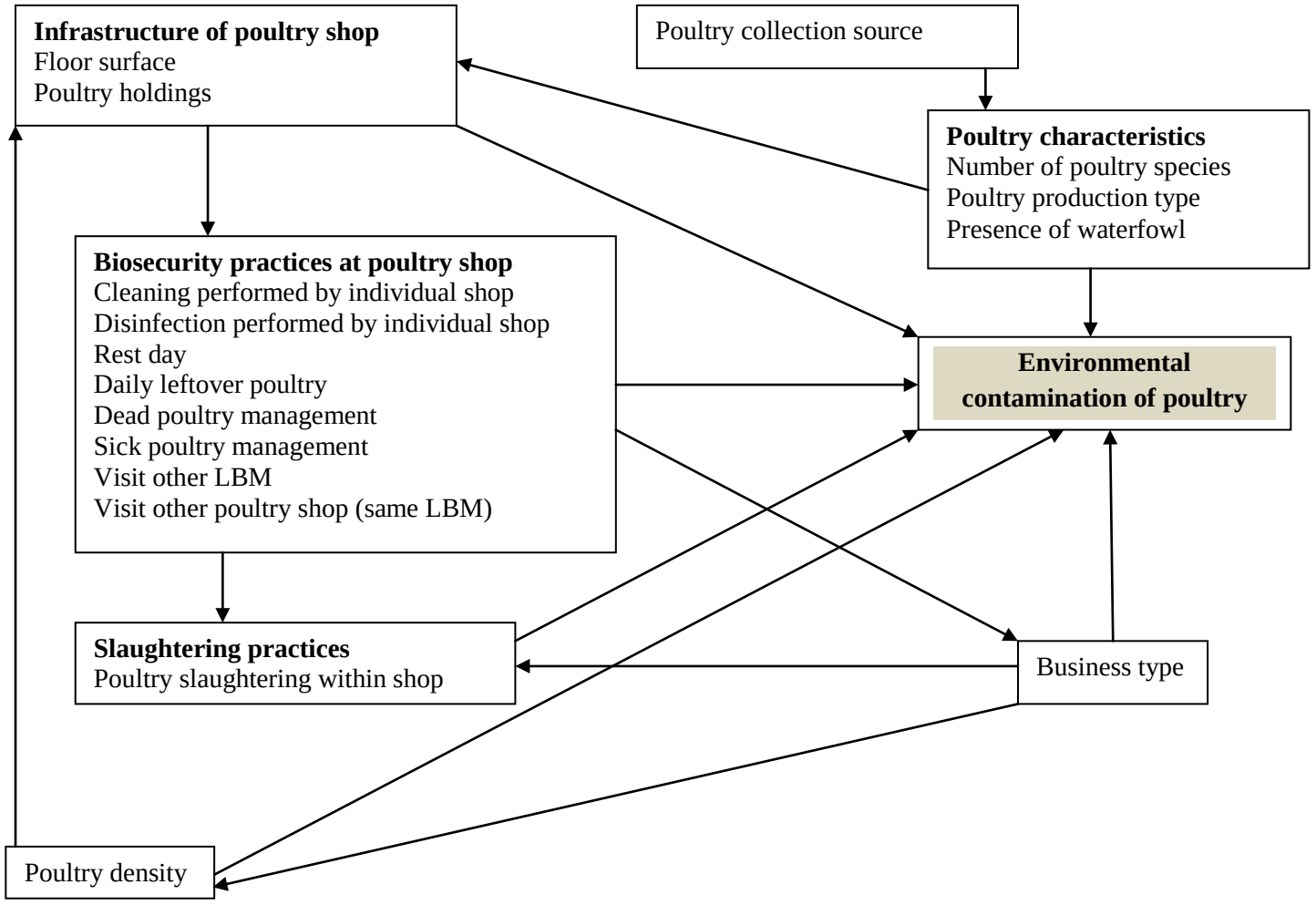

Figure 4.3. Conceptual framework for environmental contamination of individual poultry shop with avian influenza viruses

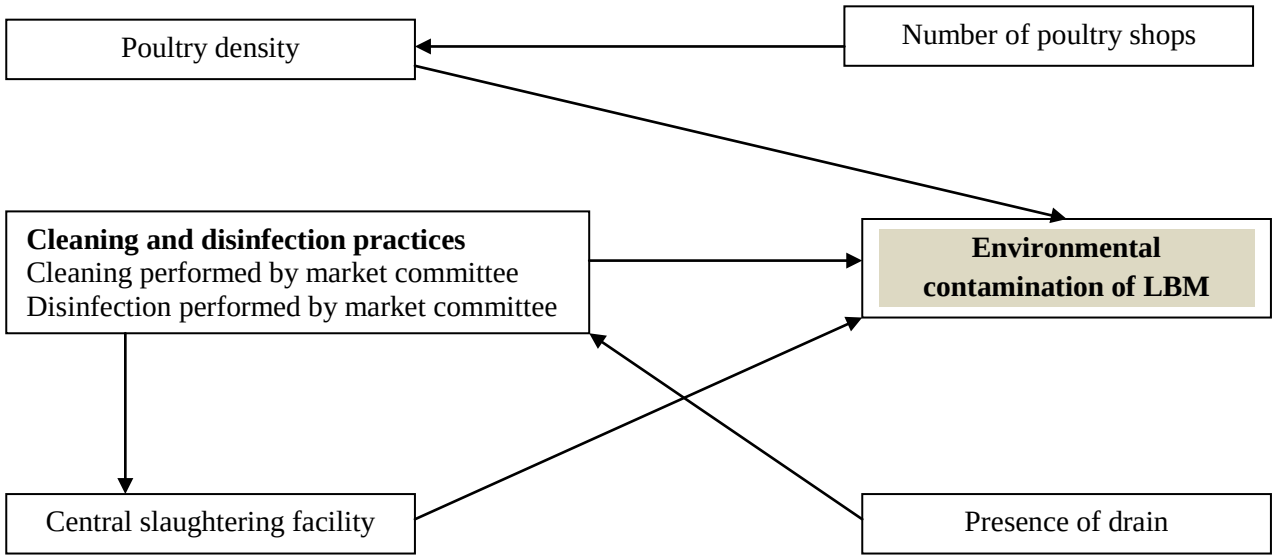

Figure 4.4. Conceptual framework for environmental contamination of LBM with avian influenza viruses

4.3.4. Shop-level biosecurity practices associated with environmental surfaces contamination by avian influenza viruses

In bi-variable analyses, poultry shops that kept backyard and commercial poultry together, held poultry in bamboo cages/floor, slaughtered poultry within the shop, had unsold poultry at the end of the previous business day, had no weekly rest day, kept sick and health poultry together, and had rough or muddy floor surfaces were more likely to harbor detectable AIV RNA in environmental specimens ($p \leq 0.05$). Poultry shops that cleaned poultry holding areas daily were found to significantly protect against environmental contamination of AI. Weekly disinfection practices were not associated with environmental contamination (Table 4.3).

In the final multivariable analysis model, poultry shops that slaughtered poultry within their shop (APR 1.6, CI: 1.1-2.3) and/or shops with unsold poultry from the previous day (APR 1.9, CI: 1.3-2.8) and/or shops that had no weekly rest day (APR 1.2, CI: 1.1-1.4) and/or shops that kept sick and healthy poultry together (APR 1.2, CI: 1.0-1.4) were more likely to harbor detectable AIV RNA in environmental specimens than their counterparts (Table 4.3). Existing cleaning and disinfection practices were not found to be protective against environmental contamination.

4.3.5. LBM-level biosecurity practices associated with environmental surface contamination by avian influenza viruses

In bi-variable analyses, we did not find any market-level variables that were significantly associated with environmental surface contamination by AI (Table 4.4). No significant association was found for any market-level variables in multivariable analysis.

4.3.6. Observation findings

We conducted three-hour observations in 60 influenza A/H5 positive and 60 influenza negative shops. We did not find any significant differences in cleaning and disinfection practices between positive and negative shops. We found that 85% of positive shops and 86% of negative shops performed daily cleaning. Among the positive shops, only 2% performed disinfection, whereas only 3% of negative shops performed disinfection during our observations.

4.4. Discussion

The evaluation of existing biosecurity practices is necessary to develop control measures for reducing the load and spread of AI in LBMs. Our study has provided a detailed understanding about the infrastructure of poultry shops and their current biosecurity practices. We identified certain biosecurity practices (slaughter of poultry within shops, leftover unsold poultry from the previous day, absence of a weekly rest day and keeping sick and healthy poultry together) that were significantly associated with environmental contamination. The size of LBMs in Bangladesh is quite variable (ranging from 10 to 55 poultry shops) in comparison to Hong Kong where the number of poultry shops in each LBM was 3-24 (Lau et al., 2007).

Poultry markets were identified as an important place for AIVs to thrive (Kung et al., 2007; Santhia et al., 2009). A previous study indicated that infected poultry may contaminate the environment of LBMs and become a source for AI transmission to humans (Kung et al., 2007). Asian countries are at risk of spreading AI to poultry, humans and the environment (Kirunda et al., 2014). Studies from multiple countries reported AI in poultry specimens at different

magnitudes: studies from Vietnam detected AI in 3.2% of poultry specimens; Egypt detected H5N1 in 12.4% of LBMs; China detected H7N9 in 10% of environmental specimens from LBMs; Indonesia detected AI in 47% of LBMs; Thailand detected H5N1 in 3.1% of market poultry; and Bangladesh detected AI in 23% of poultry specimens (Abdelwhab et al., 2010; Indriani et al., 2010; Negovetich et al., 2011; Nguyen et al., 2014; Wang et al., 2015).Our study identified that more than 90% of the sampled LBMs were positive (least one shop from a LBM tested positive) for AI type A virus and 44% of sampled LBMs were positive for H5 subtype specific RNA. This higher-level prevalence could be due to the high poultry density, presence of multiple poultry species and poor biosecurity practices within the LBMs.

Our study detected H5 and H9 co-infection in 29 (4%) poultry shops. A review suggested that the coexistence of multiple subtypes could lead to genetic reassortment and the evolution of a novel subtype which would be of high public health interest (Durand et al., 2015). Our study findings suggest that market poultry carry multiple subtypes of AIVs, which may trigger genetic reassortment and evolution of new influenza strains. A previous study from Bangladesh identified a reassortant HPAI (H5N1) virus containing a H9N2-PB1 gene in poultry specimens collected from LBM (Monne et al., 2013).

Biosecurity practices including weekly rest days, depopulation and cleaning with disinfectant reduced the risk of AI detection in poultry and environmental specimens (Yuan et al., 2014). Daily waste removal was found to be protective in Indonesia (Indriani et al., 2010). Poor biosecurity practices were significantly associated with the prevalence of H7N9 in environmental specimens of LBMs in China (Wang et al., 2015). Studies from the United States reported that environmental contamination was decreased by implementing routine cleaning and disinfection (Trock et al., 2008; Bulaga et al., 2003). However, an intervention study from Bangladesh did not find any significant variation of relative risk between intervention and non-intervention markets (RR 1.1, CI 0.44-2.76) (Biswas et al., 2015). Our study identified that most shops typically did not practice disinfection and most shops performed cleaning using only water or by broom. So, existing cleaning and disinfection practices might not be effective to reduce environmental contamination within LBM.

A few epidemiological studies have described the effectiveness of weekly or monthly rest days to reduce environmental contamination of LBMs with AI in Hong Kong (Lau et al., 2007; Leung et al., 2012). The number of H7N9 human cases was significantly reduced after permanent or temporary closure of LBMs and culling poultry in China (Leung et al., 2012; Offeddu et al., 2016; Zhou et al., 2015). During the H7N9 outbreak in China, human cases reduced from 19% to 7% after the closure of LBMs (Wang et al., 2015). In this study we recorded 74% of the sampled poultry vendors opened their shops 30 days a month without following any rest days. Despite an existing government law to implement a weekly rest day in Dhaka City LBMs to stop overnight poultry storage, the majority of shop owners were reluctant to do so to continue earning income. This study finding suggests the importance of conducting further research to evaluate the effectiveness and feasibility of a weekly rest day to reduce environmental contamination with AI.

Unsold poultry can play an important role in maintaining virus circulation in the market for a long time. A review article suggested poultry remaining in the market as an important risk factor for AI in Hong Kong, China and USA (Offeddu et al., 2016). Banning overnight poultry storage reduced the H9N2 virus isolation rate significantly in chickens (84%) in China (Leung et al., 2012). In our study, the majority of poultry shops reported that they stored poultry overnight in their shops to sell the next day. A previous study from Bangladesh also found that 73% of the poultry shops kept poultry in their stalls for more than one day (Sarker et al., 2011). Findings of our study suggest that infected unsold poultry can transmit infections to the new incoming poultry, promoting further transmission of influenza viruses in the susceptible avian hosts. This study also suggests conducting further research to evaluate the effectiveness of this intervention in reducing environmental contamination with AI.

Studies conducted in Indonesia showed that slaughtering poultry within market premises was a potential risk factor for environmental contamination (Indriani et al., 2010; Samaan et al., 2011). H7N9 was also detected in swab samples collected from the surfaces of chopping boards in China (Wang et al., 2015). People from China and Bangladesh prefer to purchase live chicken that are slaughtered in the market at the time of purchase (Biswas et al., 2015; Zhou et al., 2015). Only a few poultry shops had outside slaughter facilities in Bangladesh (Biswas et al., 2015). In this study, we observed that most of the poultry shops slaughtered poultry within the shop

premises. This practice may increase the risk of AI spread to the LBM environment. Therefore, a centralized system of slaughtering poultry should be introduced to reduce market environment contamination.

A previous study from Bangladesh reported that 58% of the sick poultry were sold to consumers and 15% were consumed by poultry vendors or workers themselves (Sarker et al., 2011). In this study, 22% of poultry shops informed us that they experienced at least one sick chicken in the last two days before our market visits and 55% of shops did not separate sick poultry from healthy poultry. These types of practices could increase the risk AIVs transmission from sick poultry to healthy poultry.

Poultry market chains in urban areas of Bangladesh are very complex. Our study found that urban LBMs collect poultry from different (or multiple) sources, such as directly from farms, middlemen and/or wholesale markets. This type of complex network system may promote the spread of AI from farm to market and make interventions difficult to implement. In China, poultry trading networks among the LBMs and LBM type (i.e wholesale, retail and mixed) were significantly associated with a higher prevalence of H7N9 in poultry. This could enhance the transmission of H7N9 to humans (Zhou et al., 2015). Poultry movement between markets plays an important role for the spread of AI from one market to another (Van Kerkhove et al., 2009). A study suggested that continuous movement of birds in the market can increase AIVs transmission (Indriani et al., 2010). Our study suggests conducting a study to assess microbial risk for HPAI in poultry value chain to develop appropriate interventions to reduce AI transmission.

4.5. Study limitations

A limitation of our study is that we could not observe poultry shop activities for full days due to time constraints and limited resources. Information collected about biosecurity from poultry vendors, workers and/or market committee members through interviews may have been biased in some contexts.

4.6. Conclusion

In conclusion, the present study identified certain modifiable risk practices, including slaughtering poultry within shops, keeping poultry overnight, keeping sick and healthy poultry together, and not practicing a weekly rest day, that increase the risk of environmental contamination with AI and could be targeted for interventions. Existing cleaning and disinfection practices were neither significantly appropriated nor effective in reducing environmental contamination. Our study suggests the use of shop-level interventions such as cleaning, disinfection, weekly rest days, no overnight poultry storage and central slaughtering facilities to reduce the risk of environmental contamination. These proposed interventions should be monitored and evaluated routinely to assess their effectiveness. Poultry traders or shop owners should be trained on proper cleaning and disinfection. This study finding will be more useful to the policy makers of Bangladesh for developing and designing effective and feasible interventions to reduce environmental contamination at LBM. We also propose future research to evaluate enhanced and improved biosecurity practices along with well-structured infrastructure for reducing environmental contamination of LBMs in low resource settings.

Chapter-5: General Discussion

Highly Pathogenic Avian Influenza H5N1 has caused repeated outbreaks in poultry in Asia, Europe and Africa (OIE, 2015). Chickens are the most susceptible poultry species to HPAI H5N1, with high morbidity and a case fatality rate as high as 100% (Alexander, 2007). H5N1 has caused severe infections in human as well. Since 2003, a total of 859 human cases with H5N1 infection have been reported, with most cases arising in South Asian countries (WHO, 2018). In March 2007, HPAI H5N1 was the first reported in poultry in Bangladesh and there have been more than 550 H5N1 outbreaks reported among poultry to date, 90% of which occurred on commercial poultry farms (OIE, 2018). A total of eight human cases including one death with HPAI H5N1 infection have been so far reported in Bangladesh (WHO, 2018). Among the reported cases, three were poultry workers (Brooks et al., 2009; IEDCR, 2012a; IEDCR, 2012b; IEDCR, 2013). So, poultry workers are a high-risk professional group to get H5N1 infection from poultry. There have been many published and unpublished studies on AI investigation at LBMs in Bangladesh (Negovetich et al., 2011; Biswas et al., 2015; Brum et al., 2016). However, those studies were conducted at small scales, covering selected areas and were cross-sectional in nature. Therefore, for this PhD thesis, different data sets were produced through a 10-year long AI active surveillance (2007 to 2016), and also through a cross-sectional study across the country with the aim of having comprehensive epidemiological findings to help control AI in Bangladesh. The overall objectives of this thesis were to understand the dynamic patterns of AI circulation in poultry sold at LBMs and to understand factors associated with the risk of AI circulation. This chapter discusses the important findings obtained from the different studies (Chapters 2-4) and their implications along with limitations, conclusions and recommendations.

5.1. Prevalence of Avian Influenza Viruses Circulation

Irrespective of poultry species, the overall prevalence of AI in the present study was low (up to 6% AIV RNA and up to 3% H5 RNA) (Chapter 2). Contrarily, the prevalence was high in environmental samples (29% AIV RNA and 10% H5 RNA) (Chapter 2). These findings are well supported by many national and international AI studies and surveillances throughout the world

(Panigrahy et al., 2002; Amonsin et al., 2008; Abdelwhab et al., 2010; Negovetich et al., 2011; Pawar et al., 2012; ElMasry et al., 2017). High prevalence in environmental samples in the present study could be due to the several reasons such as continuous shedding of virus through feces by silent carrier or infected poultry, slaughtering of poultry within LBMs and absence of interventions to reduce environmental contamination (Pooling effect for environmental samples).

Waterfowl had a higher prevalence (AIV RNA 6% and H5 3%) than chickens (AIV RNA 3% and H5 1%) in the current studies (Chapter-2), which could be due to the persistence of the AI viruses in waterfowl, as a reservoir in LBMs, without showing any clinical manifestation (Kim et al., 2009b). Very high prevalence of AIV RNA and H5 was found during the winter season and in urban LBMs (Chapter-2), which is in line with the results of other studies across the world (Auewarakul, 2008; Si et al., 2009; ElMasry et al., 2017; OIE, 2017a). Seasonal peaks of HPAI in poultry were reported during winter months in southeast Asia (Park and Glass, 2007). This pattern could be due to the cooler environmental temperature, which could promote circulation of AIVs during winter season. However, these studies were not able to identify the source of AIV infection for LBM birds. This study speculated that ducks might have been infected at the source of origin of the virus during scavenging (Henning et al., 2010), and chickens could have been infected by infected ducks. While travelling to LBMs together or caged, both ducks and chickens together on poultry stalls or LBM birds can be infected by AI contaminated LMB environment (Cardona et al., 2009). Therefore, extra hygienic care and biosecurity measures in the LBMs should be intensified during AI peak winter season in particular. Carrying ducks with other poultry species during transportation and keeping ducks in the same cage as other poultry species should be avoided as much as possible. Urban LBM environments should be treated on a regular basis with effective cleaning and disinfectants (10% hydrogen-peroxide, 70% ethanol, 2% quaternary ammonium compound, 5% formaldehyde, 10% iodophors and 3% chlorines). These strategies have been successful to reduce AI level at LBM in many countries in the world such as USA, Vietnam, and Hong Kong (Bulaga et al., 2003; Kung et al., 2003; Trock et al., 2008; Fournié et al., 2013).

5.2. Distribution of Avian Influenza Subtypes and H5N1 Clades in LBMs

Nine different AIV subtypes in different hemagglutinin (HA) and neuraminidase (NA) combinations were identified in the present study (Chapter 2), which clearly intersects with the current and commonly circulating AIV subtypes of H5 (HPAI) and H9 in Bangladesh. However, H9 subtype dominated over other subtypes, particularly in chickens, which is in agreement with previous studies (Negovetich et al., 2011; Turner et al., 2017). An earlier AI sero-survey in Bangladesh reported H9N2 subtype (18% sero-prevalence) in backyard chicken (Alam et al., 2003). H7N9 was less-commonly detected in poultry of Bangladesh in the present study. The strain of H7N9 that was detected, however, was dissimilar to the Chinese H7N9 (Wang et al., 2013). Like the current studies, multiple subtypes were also reported in domestic poultry in different countries across the world. Surveillance from Korea isolated several subtypes including H9N2, H3N2 and H6N1 in poultry sold at LBM (Seo and Kim, 2004). In USA, H1, H2, H3, H4, H5, H6, H7, H9, H10 and H11 subtypes were isolated from gallinaceous birds, waterfowl and environmental specimens from the LBMs between 1993 and 2000 (Panigrahy et al., 2002). In Hong Kong LBM, H9N2 subtype was identified in poultry (Shortridge, 1999). H9N2 virus was also prevalent in poultry throughout the Middle East and Asia (Liu et al., 2003; Seo and Kim, 2004; Aamir et al., 2007; Lee et al 2010; Moosakhani et al., 2010).

The AIV subtype diversity in the current studies suggests AIVs adaptiveness in domestic poultry and therefore suggests a chance of developing new strain of AIVs through mutation, recombination and reassortment. This widespread AIV subtype diversity in domestic poultry could be due to the cross-species transmission and adaptation of AIV subtypes in different poultry species sold at LBM. Therefore, single species poultry transportation system and single species poultry stalls are highly recommended to prevent emergence of new AI strains and future pandemic AIV strain.

Our surveillance platform detected multiple clades of HPAI H5N1 viruses such as 2.2.2, 2.2.2.1, 2.3.2, 2.3.4.2, 2.3.2.1 and 2.3.2.1a. The previous H5N1 Bangladeshi clades were 2.2, 2.3.2, 2.3.1 and 2.3.2.1 in 2011 (Islam et al., 2012; Hoque et al., 2013; Mondal et al 2013), and these clades

were genetically close to clades determined in poultry of neighboring countries including India, Bhutan, Myanmar, Nepal, China and Vietnam (Mondal et al., 2013).

5.3. Factors Associated with Avian Influenza Viruses Circulation

The risk of AIV transmission increases during winter in the Northern Hemisphere (OIE, 2017a). Regardless of poultry species, the winter season was determined as a significant risk factor for HPAI infection compared with the other seasons (Chapter 2), a result that is consistent with many other earlier studies in Bangladesh and foreign countries (Munster et al., 2007; Park and Glass 2007; Si et al., 2009; Ahmed et al., 2010). Early spring was also identified as a potential risk factor for global H5N1 outbreaks in poultry, humans and wild birds (Si et al., 2009). Temperatures in early spring in overseas countries are similar to temperatures in winter in Bangladesh. Low air circulation during winter may also trigger persistence of avian influenza viruses circulation. Influenza seasonality is closely related to virus survival, host immunity and duration of infectiousness, effective contact and contact rate. Each of these three factors can be influenced by a series of seasonal stimuli like temperature, humidity rainfall and radiation (Tamerius et al., 2011). Avian influenza viruses persist in cold water for a long time (106 to 207 days days at 17ºC to 30-102 days at 28ºC) (Stallknecht et al., 1990).

In the current study (Chapter-2), among waterfowl, ducks were more likely to be positive for AIVs compared to geese (OR 3.6, 95% CI: 2.3 -5.7). These are very likely results as aquatic birds belonging to Anseriformes and Charadriiformes are recognized as natural reservoirs for AI (Stallknecht and Shane, 1988). Among commercial chicken, Cobb type chicken were more likely to be positive for AIV than other broiler, layer and breeder types (OR 9.8, 95% CI: 3.1 -31.1) in the present study. Strain specific AIV exploration among multiple poultry species for a decade is the first time in Bangladesh to our knowledge. Genetic variability between chicken types might be the cause of discrepancy of occurrence of AI. It is also quite natural to have a higher prevalence of AI in dead chicken as chicken is the most susceptible species among other poultry species (Alexander, 2000).

Environmental samples collected from urban LBMs were more positive for AI A/H5 than those collected from rural or peri-urban LBMs (OR 2.2, 95% CI: 1.7 -2.8). As there is usually dense poultry concentration and multiple poultry species with different sources of origin in urban LBMs, there is a chance to have more prevalence of AI in LBM environment. Along with dense poultry population, human population concentration is also high in urban LBMs; hence there is the potential risk of transmitting HPAI H5N1 to the human population from AI-infected LBM environment. In the present study, AI circulation in poultry and environmental samples was negatively correlated with monthly average temperature. The findings for temperature were similar to other countries' reports where most H5N1 outbreaks were detected during winter (Auewarakul, 2008; Si et al., 2009; ElMasry et al., 2017; OIE, 2017a). In this study, we found that circulation of AIVs in waterfowl occurred quite regularly in winter compared with other poultry species. This pattern could be due to the cooler environmental temperature that could promote circulation of AIVs during winter season. The association between other climate factors and AI circulation was inconsistent; sometimes showing a positive correlation and sometimes showing a negative correlation (Chapter-3). The inconsistent association between AI activity and other climate factors (humidity, precipitation and wind speed) found in this study suggests that climate factors other than temperature might not be significant factors for AI seasonality among poultry in Bangladesh.

5.4. Role of Avian Influenza Viruses for Contaminating Environment of LBMs

Almost all LBM environments were contaminated with AIVs (either by H5 or H9/other sub-types) in the present study (Chapter-4). Earlier studies also support these findings that poultry markets are an important place for AIVs (Kung et al., 2007; Santhia et al., 2009).

The current study detected AIV RNA in environmental specimens from 26% of shops (H5 in 8% shops and H9 in 14% shops and un-type in 4% shops) (Chapter-4). These results reflect correctly the findings of other studies where infected poultry may contaminate the environment of LBMs and become a source for AIV transmission to humans (Indriani et al., 2010; Kang et al., 2015; Wang et al., 2015).

Biosecurity practices including weekly rest days, depopulation and proper cleaning and disinfecting the environment reduced the risk of AIV detection in poultry and environmental specimens (Yuan et al., 2014). Poor bio-security practices were significantly associated with the prevalence of H7N9 in environmental specimens of LBMs in China (Wang et al., 2015).

Our study found that existing cleaning and disinfection practices were not protective for reducing environmental contamination, which is supported by a Bangladeshi study (Biswas et al., 2015). However, many studies found that proper cleaning and disinfection reduced environmental contamination significantly (Bulaga et al., 2003; Lau et al., 2007; Trock et al., 2008; Indriani et al., 2010; Fournié et al., 2013).

Poultry shops that slaughtered poultry within the shop and/or held poultry in bamboo cages and/or those shops that had unsold poultry at end of the business day and/or those shops with rough muddy floors were more likely to harbor more detectable AI RNA in environmental specimens in the present study (Chapter 4). These practices certainly make the LBM environment more likely to be contaminated with AIVs and create a suitable environment to spread AIVs to human (Biswas et al., 2015). It is therefore the existing biosecurity practices should be modified and improved to prevent environmental contamination and human transmission. For example, banning overnight poultry storage reduced H9N2 virus isolation rate significantly in chickens (84%) in China (Leung et al., 2012).

5.5. Limitations

My PhD research study has few limitations. The main limitation of this study is low sample size and frequency of sampling. Due to the funding constraint, we collected limited number of sample from poultry. The field team visited LBM on monthly basis to collect sample. We conducted surveillance in five of the 64 districts. Therefore, findings of my research do not represent the whole country. During poultry enrollment, we used a convenience sampling technique (non-probability sampling technique) to select poultry.

Due to the funding shortage, we were not able to test all AIV positive specimens for typing of the full set of AIV subtypes. Most of the AIV-positive specimens were further sub-typed for H5, H7 and H9 only.

As the surveillance was fully dependent on external funding, we were not always able to be consistent with the surveillance sites throughout the whole study period. Sometimes, we extended surveillance sites and increased sample size. Sometimes, we reduced surveillance sites as well as sample size. Initially, we began surveillance with waterfowl sampling only. Then we extended to other poultry species (commercial chicken and backyard chicken).

Another limitation for biosecurity study (chapter 4) was that the duration of sample. Identification of environmental contamination was based on a cross-sectional survey in which poultry shops were sample only once that did not reflect seasonality of avian influenza circulation. Due to the resource limitation, we could not test influenza positive samples for neuraminidase. Information about biosecurity collected from poultry vendors, workers and/or market committee members through interviews may have been biased to some extent though we took the utmost care. Inability to directly observe and validate the cleaning and disinfecting of premises may have led to wrong inference.

5.6. Conclusion and recommendations

Avian influenza surveillance identified AIVs including H5 and H9 subtypes in domestic duck, geese, commercial chicken and backyard chicken. H5 was the predominant subtype in waterfowl and H9 was the predominant subtype in commercial chicken.

The peak timing of AIVs occurrence was varied by poultry species types. Avian influenza viruses circulation in waterfowl followed seasonality and the peak of AIVs occurrence was mostly observed between December and January (winter months). In chicken, no clear seasonal pattern of AIVs occurrence was determined for commercial and backyard chickens.

This LBM-based surveillance was useful to detect molecular changes of AI viruses over time in poultry and may also provide sentinel detection of novel influenza viruses of public health importance.

Detection of AIVs in the LBMs suggests that LBMs may act as important place for AIV transmission, maintenance and amplification in Bangladesh.

Existing cleaning and disinfection practices were neither significantly appropriate nor effective in reducing environmental contamination. Therefore, there is a need of future works on this aspect.

This study identified certain modifiable risk practices, including slaughtering poultry within shops, keeping poultry overnight, keeping sick and healthy poultry together, and not having a weekly rest day, that increase the likelihood of risk of environmental contamination and could be targeted for future interventions.

This monthly surveillance data is crucial for both animal health and public health authorities to respond epidemics by providing interventions at LBM to mitigate AI transmission from poultry to human.

5.7. Future directions

LBM-based surveillance demonstrated that it is an important platform for sentinel detection of novel AIVs of public health importance. Therefore, LBM surveillance should be continued for a longer period. I recommend weekly sampling instead of monthly sampling from domestic poultry, wild bird and environment of poultry market.

Monitoring evolutionary changes of AIVs in poultry should be continued to detect mutation and re-assortment events. Further research is needed to characterize AIVs that can facilitate the testing and selection of pre-pandemic vaccine viruses for humans and poultry.

LBMs should be targeted to provide interventions that improve biosecurity and cleaning/disinfection to reduce AIV transmission.

Free ranged or backyard duck population should also be targeted to provide intervention.

Future research to evaluate enhanced and improved biosecurity practices along with well-structured infrastructure for reducing environmental contamination of LBMs should be undertaken.

Better understanding about AIV transmission or spread in the poultry value chain is necessary to identify high risk areas and providing interventions to reduce transmission. A study should be conducted to identify potential risk areas for AI spread or transmission in the poultry value chain through microbial risk mapping.

Future research to design and evaluate interventions for reducing environmental contamination and minimizing AIV transmission between poultry-to-poultry and poultry-to-human should be conducted.

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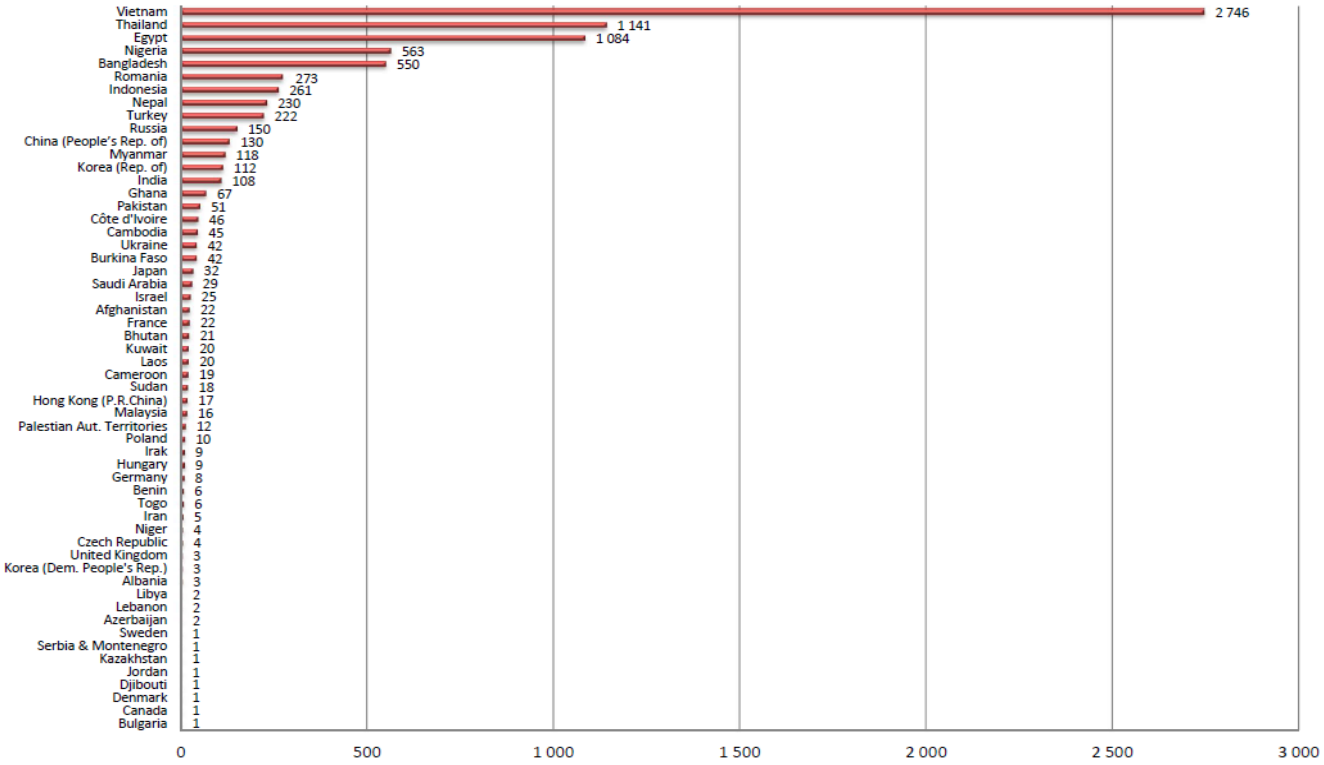
Appendices

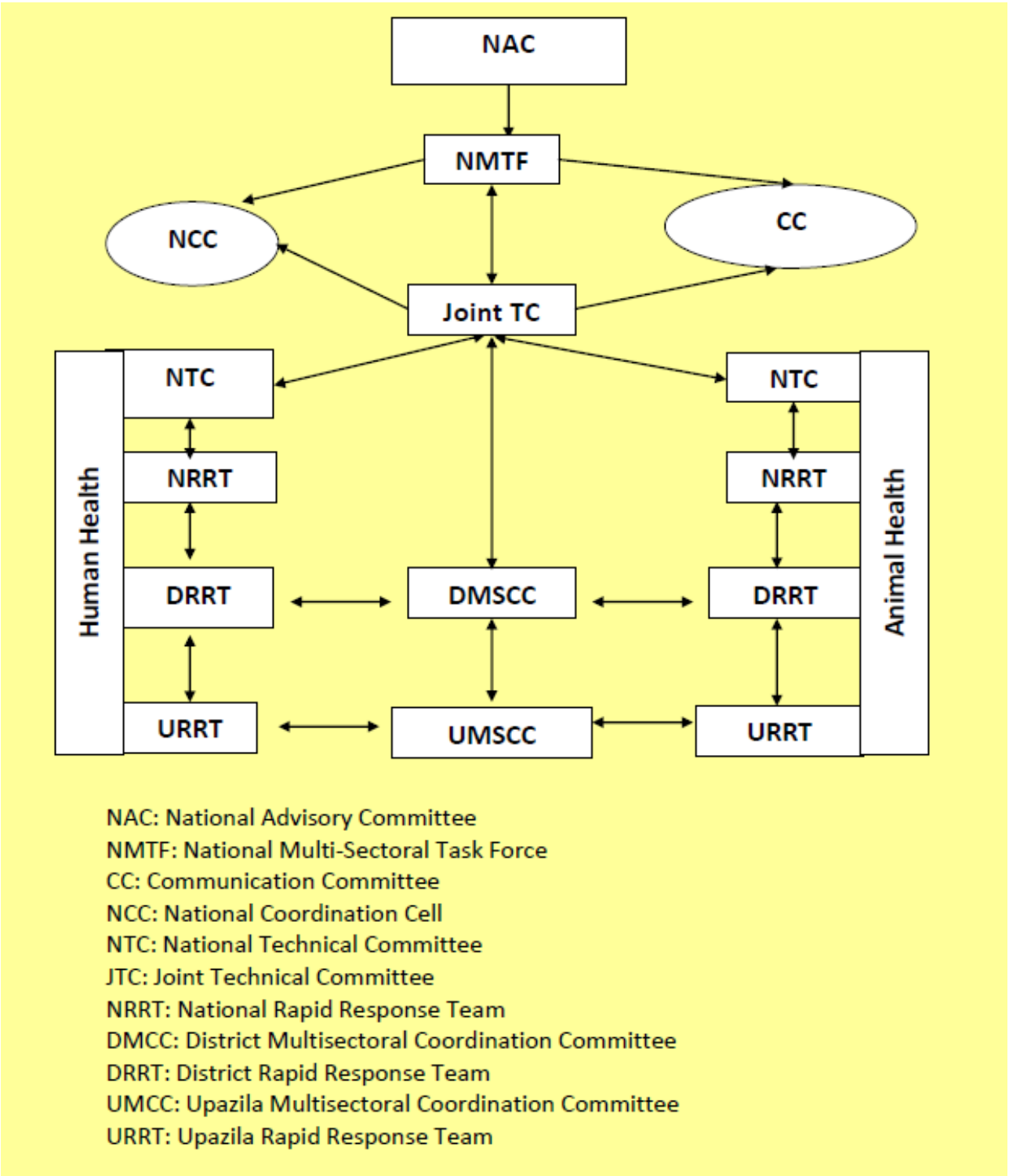
Appendix I: HPAI (H5N1) outbreaks in poultry reported to the OIE, end of 2003 to 28 November 2016 (OIE 2018)



Appendix II: Number of confirmed human cases for avian influenza A (H5N1), 2007-2017 (WHO 2018).

Country	2003-2009*		2010-2014**		2015		2016		2017		Total	
	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths	cases	deaths
Azerbaijan	8	5	0	0	0	0	0	0	0	0	8	5
Bangladesh	1	0	6	1	1	0	0	0	0	0	8	1
Cambodia	9	7	47	30	0	0	0	0	0	0	56	37
Canada	0	0	1	1	0	0	0	0	0	0	1	1
China	38	25	9	5	6	1	0	0	0	0	53	31
Djibouti	1	0	0	0	0	0	0	0	0	0	1	0
Egypt	90	27	120	50	136	39	10	3	0	0	356	119
Indonesia	162	134	35	31	2	2	0	0	0	0	199	167
Iraq	3	2	0	0	0	0	0	0	0	0	3	2
Lao People's Democratic Republic	2	2	0	0	0	0	0	0	0	0	2	2
Myanmar	1	0	0	0	0	0	0	0	0	0	1	0
Nigeria	1	1	0	0	0	0	0	0	0	0	1	1
Pakistan	3	1	0	0	0	0	0	0	0	0	3	1
Thailand	25	17	0	0	0	0	0	0	0	0	25	17
Turkey	12	4	0	0	0	0	0	0	0	0	12	4
Viet Nam	112	57	15	7	0	0	0	0	0	0	127	64
Total	**468**	**282**	**233**	**125**	**145**	**42**	**10**	**3**	**0**	**0**	**856**	**452**

Appendix III: Flow chart of committees at different levels for avian and pandemic influenza in Bangladesh



The National Advisory Committee (NAC) will be headed by the Minister of Ministry of Health and Family Welfare (MoHFW). They will endorse the National Plan before sending for approval by Ministry, monitor/review the activities under the plan. The National Multi-sectoral Task Force (NMTF) will be headed by Secretary of MoHFW. They will also endorse the National Plan before sending for approval by Cabinet and support implementation of the National Plan. The Communication Committee (CC) will be headed by the Joint Secretary (Public Health and WHO) of MoHFW who will endorse communication materials to NMFT for final approval. The Joint Technical Committee (JTC) will be headed by the Director General of Health Services. The committee will sit when required to decide matters arising from issues concerning decision of both National Technical Committee. National Technical Committees for both Health and Livestock will be headed by the Director General of Health Services and the Director General of Livestock, respectively. They will implement respective sections of the National Plan (Human health and Animal health, respectively). The District Multi-sectoral Co-ordination Committee (DMCC) will be headed by Member of Parliament in-charge of District. The committee will coordinate district AI activities. The Upazila Multi-sectoral Co-ordination Committee (UMCC) will be headed by Upazila Chairman. The committee will coordinate upazila AI activities (GoB 2011).

The surveillance focuses on early detection of AI, including novel virus both in birds and humans, rapid response, early warning and situation monitoring. Three committees such as The National Rapid Response Team (NRRT), The District Rapid Response Team (DRRT) and The Upazila Rapid Response Team (URRT) will be responsible for surveillance and outbreak investigation in both animal and human sectors (GoB 2011).

Appendix IV: Field data sheet for waterfowl

<table>
<tr><td colspan="3">Unique ID
<table><tr><td></td><td></td><td></td><td></td><td></td></tr></table></td><td colspan="3" rowspan="2">Name, address and phone number (if any) of the owner of the birds/animal:</td><td colspan="3">Date of specimen collection (dd/mm/yy)

Time of specimen collection (hh/mm) am/pm</td></tr>
<tr><td colspan="3" rowspan="2">Description of the husbandry practice:
Backyard poultry (Production sector 4) □ 1
Small scale poultry (Production sector 3) □ 2
Commercial poultry (Production sector 2) □ 3
Industrial poultry (Production sector 1) □ 4</td><td colspan="3">Sample source</td></tr>
<tr><td>Duck market sample</td><td>Rural and peri urban community sample</td><td>Private sector or NGO sample (other then partner NGO)</td></tr>
<tr><td colspan="3">Details of the vaccination program undertaken by the farm:</td><td colspan="2">Total number of birds in the farm/house before 7 days:</td><td colspan="2">Number of dead birds in last 7 days:
<table><tr><td>7</td><td>6</td><td>5</td><td>4</td><td>3</td><td>2</td><td>1</td></tr><tr><td></td><td></td><td></td><td></td><td></td><td></td><td></td></tr></table></td><td>1</td><td>2</td><td>3</td></tr>
<tr><td colspan="3"></td><td colspan="2"></td><td colspan="2">Total dead birds:</td><td colspan="3">Any treatment given to the infected birds. If yes, give details:</td></tr>
<tr><td>Type of bird (Fowl/duck/geese)</td><td>Local name of the bird</td><td>Age of the bird (Juvenile or Adult)</td><td colspan="2">Domestic (D) or wild (W)</td><td colspan="2">If wild bird, migratory (M) or resident (R)</td><td colspan="2">Health status of the bird (H=Apparently healthy, S= Sick, D=Dead)</td><td>Type of sample collected (T= Tracheal sample, C=Cloacal sample, F=Fecal specimen)</td></tr>
<tr><td></td><td></td><td></td><td colspan="2"></td><td colspan="2"></td><td colspan="2"></td><td></td></tr>
</table>

*If additional page is needed, opposite side of the page may be used.

Appendix V: Field data sheet for commercial chicken

<table>
<tr><td colspan="3">Unique ID

<table><tr><td></td><td></td><td></td><td></td><td></td></tr></table></td><td colspan="2" rowspan="5">Name of vendors:

Name of the market:

Upazilla:

District:</td><td colspan="2">Date of specimen collection (dd/mm/yy)</td><td></td></tr>
<tr><td colspan="2">Time of specimen collection (hh/mm) am/pm</td><td></td></tr>
<tr><td colspan="3">Sample source</td></tr>
<tr><td colspan="3">Type of poultry:
marking by drawing circle around number</td><td>Live bird market</td><td>Poultry shop</td><td>Farm</td></tr>
<tr><td colspan="2">Broiler</td><td>1</td><td rowspan="5">1</td><td rowspan="5">2</td><td rowspan="5">3</td></tr>
<tr><td colspan="2">Layer</td><td>2</td></tr>
<tr><td colspan="2">Breeder</td><td>3</td></tr>
<tr><td colspan="2">Cock</td><td>4</td></tr>
<tr><td colspan="2">Others, please mention…</td><td>5</td></tr>
<tr><td colspan="3">Source of poultry (insert number)</td><td></td><td colspan="3" rowspan="2">Age of the bird
(Juvenile=1, adult=2 or 3=discarded) 1 2 3 (drawing circle)</td></tr>
<tr><td colspan="3">Same Upazilla (1=Yes or 2=No)</td><td></td></tr>
<tr><td colspan="3">Same District (1=Yes or 2=No)</td><td></td><td colspan="3" rowspan="2">Average number of poultry in your shop/ day:</td></tr>
<tr><td colspan="3">If possible, please mention address of farm where poultry were reared……</td><td></td></tr>
<tr><td>Health status of the bird
(H=Apparently healthy, S= Sick, D=Dead)</td><td colspan="2">Major clinical signs</td><td colspan="3">Number of dead birds in last 2 days:</td><td colspan="2">Type of sample collected</td></tr>
<tr><td rowspan="3"></td><td colspan="2" rowspan="3"></td><td>Day 1</td><td>Day 2</td><td colspan="2" rowspan="3">T=Tracheal
C= Cloacal
CT= Mixed (tracheal and cloacal swab)
F=Fecal sample
E=Environmental pool</td></tr>
<tr><td></td><td></td></tr>
<tr><td colspan="2">Total dead birds:</td></tr>
</table>

Appendix VI: Field data sheet for backyard poultry sample

<table>
<tr><td colspan="2">Unique ID

BM - ____ - C - ____

BH - ____ - P - ____

BM=Backyard poultry from LBM
BH=Backyard poultry from household</td><td colspan="2">Name, address and phone number (if any) of the owner of the birds/animal:
Name of vendors:

Father’s/Husband’s name:

Village:

Union:

Upazilla:

District:</td><td colspan="3">Date of specimen collection (dd/mm/yy)

Time of specimen collection (hh/mm) am/pm

Sample source
Live bird market sample | Rural and peri urban community sample | Private sector or NGO sample (other than partner NGO)
1 | 2 | 3</td></tr>
<tr><td colspan="2" rowspan="2">Details of the vaccination program undertaken by the farm:</td><td rowspan="2">Total number of poultry in the farm/house today:</td><td>Number of dead poultry in last 7 days:
7 | 6 | 5 | 4 | 3 | 2 | 1</td><td colspan="3" rowspan="2">Any treatment given to the infected birds. If yes, give details:</td></tr>
<tr><td>Total dead birds:</td></tr>
<tr><td>Type of poultry
C=chicken
D=Duck
G=Geese
O=Others</td><td>Age of the bird
(Juvenile or Adult)</td><td colspan="2">Health status of the bird
(H=Apparently healthy, S= Sick, D=Dead)</td><td colspan="3">Type of sample collected
(T= Tracheal sample, C=Cloacal sample, F=Fecal specimen)</td></tr>
<tr><td></td><td></td><td colspan="2"></td><td colspan="3"></td></tr>
</table>

Appendix VII: Master mix composition, Reaction condition, Primer/Probe list

a) Master mix composition of rRT-PCR:

Reagent	Concentration	µL/rxn
PCR Buffer	2x	12.5
Agpath Enzyme mix	25x	1.0
Forward primer	40 µM	0.5
Reverse primer	40 µM	0.5
Probe	10 µM	0.5
Detection Enhancer	25x	1.0
water		4.0

5 µL of RNA was used as a template in 20 µL of master mix (Total 25 µL reaction volume).

b) Reaction condition:

	Temp	**Duration**	**Cycles**
Cycle 1	50	30 min	1
Cycle 2	95	5 min	1
Cycle 3	95	15 sec	45
	55	30 sec	collect

c) Primer/Probe list:

Target	Primer sets/Probe	Sequence (5'-3')	Reference
Matrix (Inf A)	Forward	5’-GAC CRA TCC TGT CAC CTC TGA C-3’	CDC (Only for partner lab) and WHO Influenza Lab Mannual
	Reverse	5-‘AGG GCA TTY TGG ACA AAK CGT CTA-3’	
	Probe1	5’-FAM-TGC AGT CCT CGC TCA CTG GGC ACG-BHQ1-3’	
H5a (HA)	Forward	5’TGG AAA GTR TAA RAA ACG GAA CGT-3’	CDC (Only for partner lab) and WHO Influenza Lab Mannual
	Reverse	5’-YGC TAG GGA RCT CGC CAC TG-3’	
	Probe 2 2*	5’-FAM-CAA CTA TCC GCA G”T”A TTC AGA AGA AGC AAG ATT AA-3’ Internal quencher BHQ-1 at position “T”	
	Probe 1 2*	5’-TGA CTA CCC GCA G”T”A TTC AGA AGA AGC AAG ACT AA-3’ Internal quencher BHQ-1 at position “T”	
H5b (HA)	Forward	5’-GGA ATG YCC CAA ATA TGT GAA ATC AA-3’	CDC (Only for partner lab) and WHO Influenza Lab Manual
	Reverse	5’-CCA CTC CCC TGC TCR TTG CT-3’	
	Probe2	5’-FAM-TAC CCA TAC CAA CCA “T”CT ACC ATT CCC TGC CAT-3’ Internal quencher BHQ-1 at position “T”	
H9 (HA)	Forward	5’-ATG GGG TTT GCT GCC -3’	CDC (Only for partner lab) and WHO Influenza Lab Manual
	Reverse	5’-TTA TAT ACA AAT GTT GCA YCT G-3’	
	Probe 1	5’-FAM-5’-TTCTGGGCCATGTCCAATGG-BHQ1-3’	
H7 (HA)	Forward	5’-ATT GGA CAC GAG ACG CAA TG-3’	CDC (Only for partner lab) and WHO Influenza Lab Manual
	Reverse	5’-TTC TGA GTC CGC AAG ATC TAT TG-3’	
	Probe 1	5’-FAM-TAA TGC TGA GCT GTT GGT GGC A-BHQ-3’	

Appendix VIII: List of LBMs with number of poultry shops from 10 cities

Sl. No.	Name of the market	Number of shops	City
1	AGB colony kacha bazar (Motijheel)	11	Dhaka
2	Banani kancha bazar	10	Dhaka
3	Basabo bazar	15	Dhaka
4	Bonosri kacha bazar	17	Dhaka
5	Dhupkhola kacha bazar	14	Dhaka
6	Doya gonj bazar, Gandaria	14	Dhaka
7	Fakirapul kancha bazar	15	Dhaka
8	Gabtoli boro bazar	14	Dhaka
9	Gulshan-1 market	10	Dhaka
10	Hatirpul city corporation market	18	Dhaka
11	Hazari bagh	10	Dhaka
12	Jatrabari retail market	13	Dhaka
13	Joar sahara bazar, Shewra (before Khilkhet)	12	Dhaka
14	Kaptan bazar	50	Dhaka
15	Karwan bazar	30	Dhaka
16	Kathal bagan bazar	18	Dhaka
17	Kellar Mor bazar	13	Dhaka
18	Khilgaon kacha bazar	20	Dhaka
19	Khilket bazaar	13	Dhaka
20	Kochukhet cantonment market, Mirpur 14	40	Dhaka
21	Malibag bazar	19	Dhaka
22	Mirpur 11 no kacha bazar	18	Dhaka

23	Mirpur 12 no bazar (muslim bazar)	15	Dhaka
24	Mirpur 2	13	Dhaka
25	Mirpur section 6 market (near Mirpur-2)	20	Dhaka
26	Mogbazar kancha market	15	Dhaka
27	Mohammadpur krishi market	24	Dhaka
28	Moulavi bazar	15	Dhaka
29	Nababganj bazar (near Lalbagh)	13	Dhaka
30	Nakhal para market	12	Dhaka
31	New Market kancha bazar	72	Dhaka
32	Notun bazar, Kallayanpur	12	Dhaka
33	Noya bazar	10	Dhaka
34	Palashi bazar	10	Dhaka
35	Rail-line talpotti bazar, Jurain, Shampur	25	Dhaka
36	Ray shaheb bazar (Janson Road)	13	Dhaka
37	Rayerbazar city corporation market, Dhanmondi	20	Dhaka
38	Shah ali market kancha bazar, Mirpur-1	30	Dhaka
39	Sham bazar, sadarghat	10	Dhaka
40	Shantinagar kancha bazar	13	Dhaka
41	Shipahibag bazar	10	Dhaka
42	Sonir akhara kacha bazar	20	Dhaka
43	Sukrabad bazar	15	Dhaka
44	Sutrapur	15	Dhaka
45	Taltala market, Agargaon	20	Dhaka
46	Thatari bazar	20	Dhaka
47	Town hall market, Mohammadpur	30	Dhaka

48	Uttar adabar (adabar 5 no)	12	Dhaka
49	Uttar badda bazar	10	Dhaka
50	Uttara 11 no sector chowrasta bazar	12	Dhaka
51	Uttara, Azampur market	15	Dhaka
52	15 no. Airport bazar	10	Chittagong
53	Bahardor hat	20	Chittagong
54	Bakishir hat	15	Chittagong
55	Bandor tilla kacha bazar	10	Chittagong
56	Bangla bazar, Madarbari	10	Chittagong
57	Chaktai kacha bazar	10	Chittagong
58	Chalk bazar	20	Chittagong
59	Choumuhoni bazar, Agrabad	18	Chittagong
60	Chowdhury market, Firi port moor	12	Chittagong
61	Dewan hat city corporation market	10	Chittagong
62	Fakirhat kacha bazar, bandor	12	Chittagong
63	Firingi bazar	12	Chittagong
64	Isan mistri hat (near Custom office)	10	Chittagong
65	Kalamia bazar	10	Chittagong
66	Kamal bazar, Mohora	15	Chittagong
67	Katgor steel mill bazar	15	Chittagong
68	Kazir dewri	20	Chittagong
69	Kornel hat	12	Chittagong
70	Pahartali kacha bazar	15	Chittagong
71	Reazuddin bazar	25	Chittagong
72	Kornofully market	20	Chittagong

73	Zhowtala bazar	15	Chittagong
74	Board bazar	15	Gazipur
75	Gazipur chourasta bazar	15	Gazipur
76	Gazipur pouro kancha bazar	15	Gazipur
77	Konabari bazar	15	Gazipur
78	Tongi bazar	15	Gazipur
79	Badshamia bazar (Shashan gacha)	10	Comilla
80	Chalkbazar	17	Comilla
81	Rajganj bazar	11	Comilla
82	Ranir bazar	15	Comilla
83	Baburail 1 no. boubazar	10	Narayanganj
84	Chittagong road bazar	23	Narayanganj
85	Dighi babur bazar (depo bazar)	70	Narayanganj
86	Rail station bazar	10	Narayanganj
87	Shibu market	10	Narayanganj
88	Puranbazar / borobazar	13	Barisal
89	Chuowmatha bazar	12	Barisal
90	Notun bazar	10	Barisal
91	Chitrali super market (Khalishpur)	14	Khulna
92	Doulatpur bazar	13	Khulna
93	Khulna boro bazar	13	Khulna
94	Khulna new market	10	Khulna
95	Bismilla super market (Gollamari)	10	Khulna
96	Bandor lal bazar	23	Sylhet
97	Ambor khana	21	Sylhet

98	Shibganj bazar	13	Sylhet
99	Madina market	18	Sylhet
100	Major tilla	10	Sylhet
101	Horgram bazar (court bazar)	12	Rajshahi
102	Saheb bazar	14	Rajshahi
103	Laxmipur bazar	15	Rajshahi
104	Pouro bazar	43	Rangpur

Appendix IX: Description of biosecurity practices and their plausible association with environmental contamination for avian influenza

Biosecurity practices	**Categories**	**Role in avian influenza epidemiology and environmental contamination**
Cleaning poultry holding areas	Not cleaning	Retain environmental wastes that increase likelihood for the risk of environmental contamination
	Monthly	Retain environmental wastes that increase the risk of environmental contamination
	Weekly	Remove environmental wastes that decrease the risk of environmental contamination
	Daily	Remove environmental wastes that decrease the risk of environmental contamination
Disinfecting poultry holding areas	Not disinfecting	Retain virus within environmental premises that increase the risk of environmental contamination
	Monthly	Retain virus within environmental premises that increase the risk of environmental contamination
	Weekly	Reduce environmental contamination by killing virus
Weekly rest day	Yes	Supportive for cleaning and disinfection
	No	Not supportive for cleaning and disinfection

Number of unsold poultry after the end of business day	No poultry left	Prevent amplification of avian influenza viruses
	Presence of unsold poultry	Maintain and amplify avian influenza viruses
Slaughtering poultry within shop	Yes	Increase environmental contamination
	No	Decrease environmental contamination
Separation of sick poultry from healthy flock	Yes	Prevent avian influenza transmission
	No	Increase risk for avian influenza transmission
Poultry vendors visited other LBM today	Yes	Increase risk for avian influenzaspread from one market to another market
	No	Prevent avian influenza transmission
Poultry vendors visited other poultry shop today	Yes	Increase risk for avian influenza spread from one poultry shop to another poultry shop
	No	Prevent avian influenza transmission

Appendix X: List of published abstracts

1. Sukanta Chowdhury, M Salah Uddin Khan, Md. Ziaur Rahman, Md. Enayet Hossain, Md Abu Sufian, Emily Gurley, Katharine Sturm-Ramirez, Stephen P Luby, Todd Davis and Erin D Kennedy (2018). Pattern of Highly Pathogenic Avian Influenza Virus Circulation among Domestic Poultry in Bangladesh: 2007-2017. International Conference on Emerging Infectious Diseases, 26-29 August 2018, Atlanta, USA.
2. Sukanta Chowdhury, Eduardo Azziz-Baumgartner, Susan C. Trock, Ahasanul Hoque, Nord Zeidner, Ziaur Rahman, Enayet Hossain, Syed Sayeem Uddin Ahmed, Katharine Sturm-Ramirez, Erin D. Kennedy and Emily S. Gurley (2017). Association between biosecurity measures and environmental contamination with avian influenza viruses in live bird markets, Bangladesh. 9th One Health Bangladesh Conference, 17-18 September 2017, Dhaka, Bangladesh.
3. Sukanta Chowdhury, Eduardo Azziz-Baumgartner, Susan C. Trock, Ahasanul Hoque, Nord Zeidner, Ziaur Rahman, Enayet Hossain, Syed Sayeem Uddin Ahmed, Katharine Sturm-Ramirez, Erin D. Kennedy and Emily S. Gurley (2016). Association between biosecurity measures and environmental contamination with avian influenza viruses in live bird markets, Bangladesh. 14th International Scientific Conference, 2 April 2017, Chittagong, Bangladesh.
4. Sukanta Chowdhury, Amy Molitoris, Ziaur Rahman, Enayet Hossain, Sumon Ghosh, Syed Sayeem Uddin Ahmed and Erin D. Kennedy (2017). Community based surveillance to detect avian influenza in backyard poultry in Bangladesh. The Sixth ESWI Influenza Conference, 10-13 September 2017, Riga, Latvia.
5. Sukanta Chowdhury, Eduardo Azziz-Baumgartner, Susan C. Trock, Ahasanul Hoque, Nord Zeidner, Ziaur Rahman, Enayet Hossain, Syed Sayeem Uddin Ahmed, Katharine Sturm-Ramirez, Erin D. Kennedy and Emily S. Gurley (2016). Association between biosecurity measures and environmental contamination with avian influenza viruses in live bird markets, Bangladesh. International Conference on One health EcoHealth Congress, 3-8 December 2016, Melbourne, Australia.
6. Sukanta Chowdhury, M Salah Uddin Khan, Katharine Sturm-Ramirez, Emily S. Gurley, M Z Rahman, J D Heffelfinger, Stephen P Luby, and Nord Zeidner (2015). Influenza A virus surveillance in live bird markets in Bangladesh. One Health Bangladesh Conference, 30 March 2015, Dhaka, Bangladesh.
7. Sukanta Chowdhury, Salah Uddin Khan, Md. Ziaur Rahman, Sadia Afreen, Amit K. Dey, Emily S. Gurley, Katharine Sturm-Ramirez, James D. Heffelfinger, Stephen P. Luby, and Nordin Zeidner (2013). Influenza A virus surveillance in live bird markets in Bangladesh. Options for control and prevention of Influenza conference, 4-10 September 2013, Cape Town, South Africa.

Appendix XI: List of seminar presentations

1. May 8, 2018 : Dissemination seminar on zoonotic diseases research findings: towards adaptation for field use (Presentation title: Avian influenza trend in community and live bird market: 2007-2018)
2. Apr 18, 2018 : Dissemination seminar on Influenza Surveillance in Bangladesh held at Dhaka (Presentation title: Avian influenza surveillance at human-animal interface)
3. Sep 10-Sept 13, 2017 : The Sixth ESWI Influenza Conference 2017 held at Riga, Latvia (Presentation title: Community based surveillance to detect avian influenza in backyard poultry in Bangladesh)
4. Sep 17-Sep 18, 2017 : 9th One Health Bangladesh Conference held at Dhaka (presentation title: Association between biosecurity practices and environmental contamination with avian influenza in live bird markets, Bangladesh)
5. Apr 2, 2017: 14th International Scientific Conference (ISCon XV) held at Chittagong Veterinary and Animal Sciences University (Presentation title: Association between biosecurity practices and environmental contamination with avian influenza in live bird markets, Bangladesh)
6. Dec 3-Dec 7, 2016 : International Conference on One health EcoHealth Congress 2016 held at Melbourne, Australia (Presentation title: Association between biosecurity measures and environmental contamination with avian influenza viruses in live bird markets, Bangladesh)
7. August 8, 2016 : Avian Influenza Dissemination Seminar held at icddr,b (Presentation title: Avian influenza surveillance in poultry)
8. May 31-Jun 06, 2015 : Workshop on International One Health: Conservation Medicine Policy and Practice organized by EcoHealth Alliance held at Tufts University Cummings School of Veterinary Medicine, USA (Presentation title: Avian influenza surveillance in Bangladesh)
9. Mar 30- Mar 31, 2015: One Health Bangladesh Conference held at Dhaka (Presentation title: Influenza A virus surveillance in live bird markets in Bangladesh)
10. June 30 - July 03, 2014 : Workshop on Multinational Influenza Seasonal Mortality Study held at to Washington DC, U.S (Presentation title: Influenza A virus surveillance in live bird markets in Bangladesh)
11. Sep 4-Sep 10, 2013: Options for control and prevention of Influenza conference held at Cape Town, South Africa (Presentation title: Influenza A virus surveillance in live bird markets in Bangladesh)

Brief Bio-data of Sukanta Chowdhury

Sukanta Chowdhury received the Doctor of Veterinary Medicine (DVM) degree from the University of Chittagong in 2005 and Masters Degree (Medicine) from Bangladesh Agricultural University in 2007. Dr Chowdhury is currently a PhD student of epidemiology at Chittagong Veterinary and Animal Sciences University. Dr Chowdhury started his research career at icddr,b in 2009 as a Research Fellow. Dr Chowdhury is now working as an assistant scientist at icddr,b and he is the lead investigator for avian influenza surveillance platform at icddr,b. His research interests focuses on epidemiology of infectious diseases at the animal-human interface and One Health. He is involved in several research projects including avian influenza, henipavirus, anthrax, brucella, food safety, campylobacteriosis and antimicrobial resistance (AMR). Dr Chowdhury has published few research articles in the peer-reviewed journal as a first author and co-author.