EPIDEMIOLOGICAL ASSESSMENT OF HYGIENIC CONDITIONS OF LIVE BIRD MARKETS ON AVIAN INFLUENZA IN CHITTAGONG METROPOLITAN CITY, BANGLADESH

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Roll No.: 0214/04
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A thesis submitted in the partial fulfillment of the requirements for the degree of Master of Science in Epidemiology

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JUNE 2016

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Md. Abu Sayeed

June, 2016

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This is to certify that we have examined the above Master's thesis and have found that is complete and satisfactory in all respects, and that all revisions required by the thesis examination committee have been made

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Dedication

I dedicate this MS research work
to my beloved parents and brother

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List of Abbreviations

Abbreviation	Elaboration
AI	Avian Influenza
AIV	Avian Influenza Virus
AAHL	Australian Animal Health Laboratory
BALZAC	Behavioral Adaptations in Live Poultry Trading and Farming Systems and Zoonoses Control in Bangladesh
BC	Broiler Chicken
B. Com	Bachelor of Commerce
BLRI	Bangladesh Livestock Research Institute
CCP	Critical Control Point
CI	Confidence Interval
D	Duck
DC	Deshi (indigenous) Chicken
DNA	Deoxyribonucleic Acid
FAO	Food and Agriculture Organization
GEE	Generalized Estimating Equation
HPAI	Highly Pathogenic Avian Influenza
HSC	Higher Secondary Certificate
ICDDR'B	International Centre for Diarrhoeal Disease Research, Bangladesh
IEDCR	Institute of Epidemiology, Disease Control and Research
LBM	Live Bird Market
LC	Layer Chicken
LPAI	Low Pathogenic Avian Influenza
LRT	Likelihood Ratio Test
mL	Milliliter
NRL-AI	National Reference Laboratory for Avian Influenza
OR	Odds Ratio
P	Pigeon
ppm	Parts Per Million
PRTC	Poultry Research and Training Center
Q	Quail
rRT-PCR	Real Time Reverse Transcriptase Polymerase Chain Reaction
rpm	Rotation Per Minute
RNA	Ribonucleic Acid
SE	Standard Error
SSC	Secondary School Certificate
UK	United Kingdom
%	Percentage
µl	Micro liter

Abstract

Live Bird Market (LBM) is a place where live bird traders and consumers are interacted in respect to live bird trading. The environment of LBM might be contaminated with various infectious diseases like Avian Influenza Virus. No systematic study has previously been attempted for investigation of Avian Influenza status in LBM which could pose threats to the economy and public health. Therefore, a cross sectional study was conducted on hygienic status and Avian Influenza in LBMs under Chittagong Metro in Bangladesh. The overall objective of the study was to assess the LBM demographic information and hygienic status in contrast with AI prevalence followed by subtype distribution and associated risk factors.

A total of 290 pooled environmental samples along with questionnaire based identity information, participant's demography, market structure followed by management and hygienic status based data were obtained from 290 stalls under 40 different LBMs. At each stall swab samples were collected from up to 9 different sites. The samples were evaluated by Real time Reverse Transcriptase Polymerase Chain Reaction (rRT-PCR) for detection of M gene followed by subtypes of H5, H7 and H9.

Three quarters of poultry stalls were retail type where the birds were aggregated from multiple sources. Most of the stalls were closed together. There was no space provisioned for sick birds, however the stalls had access to resident wild birds. Around one third of stalls' floor was constructed using mud and more than 50% stalls used only water for cleaning the stalls. Stall vendors had predominantly I-IX level of education. The vendors had slaughtered their poultry within the stalls and the unsold birds were allowed to stay overnight in the stall for next day selling. The wastes were generally disposed into open dustbin, drain, water bodies and beside the highway or another open space.

The prevalence of AIV in LBM was 40% (95% CI: 20-60%; N=40) whereas the prevalence of avian influenza was 20.3% (95% CI: 10-30%, N=290) at stall level. Again, the prevalence of H5, H7 and H9 at stall level was 2.8% (95% CI: 1-5%), 0%

and 3.1% (95% CI: 1-6%), respectively. The prevalence of un-typed AIV was 15.2% (95% CI: 10-19%).

Fisher's exact test followed by Generalized Estimating Equation was applied to identify potential risk factors associated with Avian Influenza in LBMs. Selling of species (OR=2.5: Chicken and non-duck species versus Duck with other species.), Bird holding area (OR=1.9: Cage versus Floor) and Hygienic score (OR=3.1: Score 3 or more versus score less than 3) were identified as the risk factors for AI in LBMs. The present study has been identified the risk factors associated with the occurrence of AIV at stall level of LBMs.

Knowledge obtained from this study could provide new understanding of the distribution and transmission of AI through LBM in Bangladesh. The findings could be used to develop a proof based programme concerning environmental sanitation along with development of a strong surveillance system to reduce the AI transmission through LBMs in Bangladesh.

Keywords: Live Bird Market, Prevalence, Risk factors, Avian Influenza

Chapter-I: Introduction

Live bird market (LBM) is a place where live bird trading occurs. People usually purchase live poultry which is then slaughtered by the stall employee and dressed for consumers. In Bangladesh, LBM is the major source of retail poultry supplying to consumers. Majority of LBMs in this country are located besides road, under the tree or open sky intermingling with passerby, feral birds and street dogs.

However, metropolitan city LBMs like Chittagong LBMs are usually located in open places or in government authorized markets where both retail and wholesale trading is practiced. In LBMs poultry vendors receive different species of birds from a wide range of sources across the country through middlemen daily. Retailer sells out birds directly to end consumers, whereas wholesalers sell out their birds to retailers, hawkers and even directly to restaurant and fast food shops. Similar live bird trading occurs in other South Asian countries including India, Myanmar etc (Landes et al., 2004; Anon, 2016b).

In Thailand and Vietnam vendors usually put the dressed birds hanging in the shops (Amonsin et al., 2008) which is uncommon in Bangladesh.

As poultry enters into LBMs (particularly city LBMs) from different sources through a complex transaction chain, so infectious diseases like avian influenza can easily introduce to LBMs, amplify organisms in the market environment and disseminate across different population (bird-bird-human) (Cardona et al., 2009). Earlier studies across the world also support that LBMs are hubs of Avian Influenza Virus for amplifying and dissemination (Indriani et al., 2010a; Magalhães et al., 2010; Fournié et al., 2012b). There is also an evidence of spread of AIV from LBM to larger commercial farms and turkey operations in Pennsylvania (Trock et al., 1997).

In Bangladesh small scale LBMs are commonly seen in every town, but wholesale and big retail LBMs are specially located in larger cities, predominantly in Dhaka and Chittagong. Till now only a few studies have been conducted on LBMs of Bangladesh, whereas most of them were based on some selective LBMs under specific cities. The birds of the LBMs under Chittagong metropolitan area are aggregated from rural areas of Chittagong district as well as north and northeast

districts of the country (Hoque et al., 2014). Previously some sporadic cross-sectional studies on avian influenza have been carried out covering only small fraction of LBMs in Chittagong (Biswas et al., 2015). The present study has therefore been considered all LBMs under Chittagong Metropolitan area to assess the status of avian influenza.

Avian Influenza is a viral disease caused by Type A influenza virus under Orthomyxoviridae family. This is a negative strand RNA virus, consisting of 8 segments which are encoded up by 11 viral proteins (Marsh et al., 2008). This virus has been classified based on its surface proteins hemagglutinin (H) and neuraminidase (N). There are 16 H and 9 N subtypes in different combinations (Fouchier and Munster, 2009). Avian Influenza viruses have been reported in more than 90 species of birds (Alexander, 2000) and wild aquatic birds belonging to Anseriformes and Charadriiformes act as the major natural reservoirs for AIV (Webster et al., 1992; Fouchier and Munster, 2009). Highly pathogenic avian influenza H5N1 is a serious concern for Bangladesh. As of April 2016, 583 outbreaks (98.1% was domestic chicken and other domestic birds like pigeon, quail, duck etc.; 0.3% was crow, 0.2% was domestic swine and 1.4% was unspecified bird species) have been reported in 51 districts (N=64) since in 2007 (Biswas et al., 2008b; Monne et al., 2013; FAO, 2016). Of 8 human HPAI H5N1 cases one has been fatal in this country and it has been evidenced that affected chicken was the sources of the human cases (WHO, 2016). Along with the non-pathogenic H9N2 subtype, other viral subtype like H1N2, H1N3, H3N6, H4N2, H5N1, and H10N7 have also been reported in LBMs in Bangladesh (Negovetich et al., 2011a).

Many earlier Avian Influenza studies used live bird samples (cloacal and oropharyngeal swabs) to evaluate avian influenza status in LBMs (Wang et al., 2006; Garber et al., 2007; Cardona et al., 2009), however the present study has used environmental pool samples to evaluate AI at poultry stall and market levels.

Published and unpublished data suggested the overall subtype specific AI prevalence at stall level was 26 % AI, 4% H5 and 14% H9; however, in stalls under LBMs of different cities in Bangladesh as follows: In Dhaka, 29 % AI, 7% H5 and 13% H9 and 12% AI, 1% H5 and 5% H9 in Chittagong, 28% AI, 2% H5 and 18% H9 in Gazipur. (Sukanta Chowdhury, ICDDR'B, Personal Communication, 2016). Again

another study reported that 13.5% LBMs was positive for AIV in Bangladesh where 9.4% was HPAI H5 subtype and 1.6 % was LPAI H9 subtype (Biswas et al., 2015). Like Bangladesh similar AI prevalence in LBMs has been reported in Indonesia (47%) and the H5 subtype prevalence in Vietnam (32.2%) (Indriani et al., 2010b; Nguyen et al., 2014). The current study aims to explore true estimate of AI prevalence and the H5, H7 and H9 specific prevalence at poultry stalls and market level considering all LBMs in Chittagong Metropolitan city.

Many previous studies have explored risk factors associated with the occurrence of AI at stall level of LBMs in different parts of the world including Bangladesh. They have included mixing of several species in the same cage (OR=2.92, 95% CI: 0.9–8.7), slaughtering in the market (OR=3.5, 95% CI: 0.9-13.9), selling of duck along with other species (p=0.039) (Zhou et al., 2015) and bird holding area etc (Indriani et al., 2010b) .

It is reported earlier that the level of environmental contamination by AIV can be decreased with routine cleaning and disinfection (Indriani et al., 2010b). Proper hygienic measures should be taken in all LBMs, otherwise healthy birds that come into market everyday may become infected and persons who work or come to visit in the market may also be exposed to contaminated environment. So, this study has been conducted for knowing the overall hygienic status of LBMs under Chittagong metropolitan area.

1.1. Objectives

The objectives of the present study were as follows:

1. To evaluate the LBM demography and hygienic status in Chittagong Metropolitan City
2. To estimate the prevalence of avian influenza virus and the selective subtypes of H5, H7 and H9 at stall and LBM levels in Chittagong Metropolitan City
3. To determine potential risk factors associated with the occurrence avian influenza at stalls of LBMs in Chittagong Metropolitan City

1.2. Anticipated Outcomes

The anticipated outcomes were as follows:

1. Identified hygienic status of LBMs in Chittagong Metropolitan City
2. Estimated overall prevalence of avian influenza and the selective subtypes of H5, H7 and H9 at LBMs in Chittagong Metropolitan City
3. Determined potential risk factors associated with avian influenza in stalls of LBMs of Chittagong Metropolitan City

Chapter II: Review of Literature

Pertinent literatures on LBM demography, avian influenza, viral transmission process, outbreaks, zoonotic significance, LBM and avian influenza virus, surveillance system, prevalence of avian influenza and its subtypes, economic consequence, diagnostic approach and prevention and control techniques have been reviewed in this chapter. The fundamental motivation behind this part is to give up to date logical data in the light of past studies and as needs to identify the scientific gap and justify the present Master's research on hygienic status and status of avian influenza virus at LBM level. The review findings of important distributed and non distributed articles have been introduced under the accompanying headings as beneath.

2.1. Live Bird Market

Live bird market (LBM) is a place where people gather together to purchase live poultry for consumption. "Live poultry" indicates finished birds which are intended to be slaughtered and eaten by the end user (Fournié et al., 2012b). LBM is very popular and essential for poultry marketing throughout the world especially for developing countries like Bangladesh (Cardona et al., 2009).

In Bangladesh LBMs are either a part of a city block or a food market where other foodstuffs are traded. Live Bird Markets are variable size, having retail shops only or both retail and wholesale shops. In LBM retailer sells live or dressed birds directly to end consumers, whereas wholesalers sell out their birds to retailers, hawkers, directly to restaurant and fast food chain shops. Like India and China trading of frozen or chilled poultry is not very attractive in this country (Landes et al., 2004; Peiris et al., 2015). Again in Thailand and Vietnam vendors usually put the dressed birds hanging in the stalls (Amonsin et al., 2008) which is uncommon in Bangladesh.

Poor sanitary conditions in city LBMs like slaughtering within the stall, lack of waste disposal facility, lack of disinfectant supply, lack of regular investigation by the public health authority etc. are very common in Bangladesh which is similar to Myanmar, Pakistan and India (Anon, 2016c).

However, in Nepal the LBM traders are offered space to conduct business in a well constructed market having clean water, neat and clean toilets and freezer facilities.

They also assign doctors to inspect the birds in the market also for advocacy to reduce the risk to public health and spread of poultry diseases (Wisedchanwet et al., 2011).

In Myanmar one well structured LBM is recently introduced which handle over 100,000 birds/day where over 50,000 birds/day is manually slaughtered at site (during 6-8h/day) (Anon, 2016b).

In many of the cities of India LBM is separated from other markets (such as food, vegetables), dissimilar to Bangladesh, for broiler chicken trading where birds are slaughtered and dressed for customers in retail shops. However, water supply and waste disposal system is poor. In contrast city LBM belonging to Delhi are well structured with enough facilities like water supply, waste disposal etc. (Landes et al., 2004).

In Thailand and Vietnam most of the LBMs have permanent structures where different type of birds are traded by hanging of the dressed birds in the stall (Amonsin et al., 2008; Cardona et al., 2009).

In the aforementioned countries including Bangladesh live birds come from origin of sources to LBMs and then to consumers through a complex transmission chain and LBMs are evidenced to be hub for introducing and amplifying and transmitting of infectious diseases like Avian Influenza (Kung et al., 2003b; Senne et al., 2003; Kung et al., 2007b; ben Embarek et al., 2009)

2.2. Avian Influenza

Avian Influenza is a viral disease caused by Type A influenza virus under Orthomyxoviridae family. This is a negative stranded RNA virus and has 8 segments. The virus has been classified based on its surface proteins hemagglutinin (H) and neuraminidase (N). There are 16 H and 9 N subtypes in different combinations (Fouchier and Munster, 2009). Over 100 years ago this disease was firstly identified in Italy (Alexander, 2003). Influenza viruses have been reported in more than 90 species of birds including domestic birds (Alexander, 2000). However, wild aquatic birds belonging to Anseriformes and Charadriiformes act as the major natural reservoirs for AIV. This virus crosses species barrier to human dog, pigs, horses, harbour seals, whales, mink etc. Based on pathogenicity the AIV has 2 types: high

pathogenic avian influenza (HPAI) and low pathogenic avian influenza (LPAI). The HPAI (like H5N1, H7N7 etc) infection in poultry is characterized by sudden onset of disease followed by severe illness and sudden death. The common lesions include cyanosis and edema of the head, followed by comb, wattle, and snood (In case of turkey). The shanks of the affected birds become edematous and red discoloration. Greenish diarrhea is found in case of severely affected birds. Luckily survived birds may develop torticollis, opisthotonos, incoordination, paralysis, and drooping wings (Swayne and Suarez, 2000).

Low pathogenic (such as H9N2) infection causes decrease egg production, misshapen eggs, decrease fertility or hatchability of the eggs, respiratory signs (sneezing, coughing, ocular and nasal discharge and swollen infra-orbital sinuses), lethargy, decreased feed and water consumption, or somewhat increased flock mortality rates may be seen in chickens as well as in turkeys.

2.3. Transmission of Avian Influenza

Avian influenza viruses can be transmitted from bird to bird through direct contact. At the same time the virus can be spread out indirectly by exposure to contaminated fecal material, aerosols, water, feed, bedding materials and utensils (de Jong and Hien, 2006). If poultry traders trade infected poultry or share contaminated equipment, then the virus can be mixed and easily transmitted in poultry and LBM environment (Fournié et al., 2013). However, earlier studies reported that multiple poultry species from backyard and commercial production systems are sold together in LBM which may increase the risk of cross-species avian influenza virus transmission (Woo et al., 2006; Wisedchanwet et al., 2011).

2.4. Outbreaks of Avian Influenza

In Asia, Europe and Africa a large number of outbreaks in poultry has been recorded by high pathogenic avian influenza (HPAI) (Fournié et al., 2012a). Since the appearance of AIV in 1996 in a domestic goose in Guangdong Province, People's Republic of China, the HPAI has repeatedly been portrayed as the most prominent emerging zoonotic disease threat for humanity (Weber and Stilianakis, 2007). At the very beginning of July 2005, HPAI H5N1 viruses were rapidly expanded from China and

spreaded to the Middle East, Africa, and Europe (Yingst et al., 2006). Recently H5N1 has caused HPAI disease among many countries of the world. According to FAO (FAO, 2016) until 16 April 2016, 19,776 number of outbreak due to HPAI H5N1 has been reported throughout the world. Among which 17,958 in domestic birds include chicken, duck, quail, turkey, geese etc. and only 9 in captive birds. In wild birds until April 2016 a total of 995 number HPAI H5N1of outbreaks has been reported throughout the world. Again the 842 number of outbreaks in human population due to HPAI H5N1 has also been recorded in the world. (FAO, 2016)

In Bangladesh AI was first identified in 2007 (Biswas et al., 2008b) and now it has spread to at least 51 of 64 districts of the country. As of April 16 2016 around 583 outbreaks have been recorded of which 98.1% was domestic chicken and other domestic birds like pigeon, quail, duck etc.; 0.3% was crow; 0.2% was domestic swine and 1.4% was unspecified bird species (FAO, 2016).

Among the Asian countries the highest number of outbreaks due to HPAI H5N1 has been recorded in Indonesia (N=5,092) among which 96.1% was domestic birds including chicken, duck, pigeon, turkey and quail; 0.04% was wild bird and 3.9% was not specified (FAO, 2016).

In India the initial evidence of rapid spread of the HPAI H5N1 among the different states was found in 2008 where market birds constitute the major percent of infected birds followed by domestic birds and wild birds (Rao, 2008). If market birds become infected, then the virus would be able to spell back to the farms more easily and viral mutation can be occurred to produce new strain. Till 16 April, 2016 the number of recorded outbreaks in India and Bhutan was 159 and 21 respectively. Again the first serologic evidence of AI has been found in Nepal in October 2005 (Pant and Selleck, 2007) and till now 125 outbreaks due to HPAI H5N1 has been recorded in Nepal. According to Food and Agriculture Organization (FAO) the H5N1 is still circulating in several countries of Asia and Africa. They consider H5N1 viruses endemic in poultry in six countries including Bangladesh, People's Republic of China, Egypt, India, Indonesia and Vietnam. Along with the HPAI H5N1 a total number of 1,660 outbreaks by H7 (including High and Low pathogenic form of H7N1, H7N2, H7N3, H7N6, H7N7, H7N8 and H7N9) and 1,619 by H9 subtype (including LPAI H9N1 and H9N2) has been reported throughout the world (FAO, 2016).

Several earlier studies have been shown that LBMs act as a hub for the circulation of AIV in many south Asian countries including China, Vietnam, Indonesia etc. (Indriani et al., 2010a; Magalhães et al., 2010; Fournié et al., 2012b). During the outbreak farmers' natural intention are to sell out their birds as quick as possible to minimize economic impact. So, the virus can be spread out in all the stalls of LBMs and can contaminate LBM environment as well as pose potential threat for human population.

Like Hong Kong if the AIV can establish in the LBM (Webster, 1998), then it can easily mutate and produce new strain not only that through spell back process it can be spread out in the commercial farms as well as among the backyard chickens. In recent years, a number of repeated outbreaks of both HPAI and LPAI have been reported in Europe and North America which were associated with rare infections among humans exposed to infected poultry (Fouchier et al., 2004; Herlocher et al., 2004; Hirst et al., 2004; Koopmans et al., 2004; Tweed et al., 2004). So, LBM has a potential consequence in the chain of AIV transmission.

2.5. Occurrence of HPAI Human Cases

Since 2003 a total of 650 human cases with HPAI H5N1 infection have been reported in the world, however more dominating in Asian countries (Senne et al., 2003).

Epidemiological studies have found that most human cases with HPAI H5N1 infection had a history of poultry exposure including direct handling, slaughtering and consumption of dead or sick poultry (Mounts et al., 1999; Chan, 2002; Beigel et al., 2005; Dinh et al., 2006).

Till now the highest number of human infection by H5N1 has been reported in Egypt (350), whereas the highest number of deaths occurred in Indonesia (165) (WHO, 2016). In China the LBM has been identified as the main risk factor for Avian Influenza (Dinh et al., 2006).

In Bangladesh one fatal and six non-fatal HPAI H5N1 cases have been reported so far of which three of them were poultry workers of Dhaka city LBMs (Senne et al., 2003; Brooks et al., 2009; IEDCR, 2013; Haider et al., 2015).

According to molecular analysis of HPAI H5N1 3 different clades are perpetuating in poultry, wild bird and human population in Bangladesh: clade 2.2, clade 2.3.4 and clade 2.3.2.1 (Hoque et al. 2014). These Bangladeshi HPAI H5N1 clades are close congeners to the clades of Asian countries (Jiang et al., 2010; Islam et al., 2012; Hoque et al., 2013; Marinova-Petkova et al., 2014). Re assortment was reported to occur between 2.3.2.1 and 2.3.4.2 in Bangladesh (Gerloff et al., 2014).

2.6. Avian Influenza at LBM and Associated Factors

Highly Pathogenic Avian Influenza virus has been identified from LBMs of many Asian countries like China, Vietnam, Thailand, Indonesia, Egypt, and Cambodia which is suggesting that LBMs could be a potential source for H5N1 infection among poultry and humans (Nguyen et al., 2005; Amonsin et al., 2008; Abdelwhab et al., 2010; Indriani et al., 2010a; Wan et al., 2011; Leung et al., 2012). Both HPAI and LPAI subtypes have been identified from LBMs in Bangladesh (Pant and Selleck, 2007; Negovetich et al., 2011a; Gerloff et al., 2014; Biswas et al., 2015).

Many earlier studies conducted in Bangladesh have been suggested the overall estimated AI and subtype specific AI prevalence at stall level were 26% AI, 4% H5 and 14% H9. However, the prevalence of AI in LBMs according to geographical areas was as follows: In Dhaka, 29% AI, 7% H5 and 13% H9; in Chittagong, 12% AI, 1% H5 and 5% H9; in Gazipur 28% AI, 2% H5 and 18% H9 (Sukanta Chowdhury, ICDDR'B, Personal communication, 2016). Biswas et al. (2015) has been reported the AI prevalence at market level was 13.5% of which the H5 prevalence was 9.4% and the H9 prevalence was 1.6 % in Dhaka and Chittagong of Bangladesh combined. The prevalence of LPAI H9N2 in birds was also reported to be 16.5% at LBMs in this country (Negovetich et al., 2011a).

In contrast high prevalence in LBMs has been reported in Indonesia (AI 47% and H5 32%) (Indriani et al., 2010b; Nguyen et al., 2014), Vietnam (H5 32.2%), South Korea (AI 10% and H9N2 6.8%), (Lee et al., 2010), Hong Kong (H9N2 4.4% (Shortridge, 1999), and Egypt (12.4–40.8% H5) (Abdelwhab et al., 2010). The presence of H1N2, H1N3, H3N6, H4N2 and H10N7 was also identified from LBMs of Bangladesh (Negovetich et al., 2011a).

There are several risk factors for LBM contamination by AI including overnight housing of unsold poultry (Bulaga et al., 2003), selling of duck along with other species (p=0.039) (Zhou et al., 2015), Mixing of several species in the same cage (OR=2.92, 95% CI: 0.9–8.7), slaughtering within the market stalls (OR=3.5, 95% CI: 0.9-13.9), market without rest day (prevalence of AI before rest day 8.9% and after rest day 1.7%) (Kung et al., 2003a), trading types (retail and wholesale both have importance for virus amplification; retail, wholesale or mixed: p=0.046) (Peiris et al., 2015; Zhou et al., 2015), market disinfectant, wild bird contamination etc. Here some of the risk factors have been expressed in odd and prevalence, whereas some have been expressed through univariate p-value in respect to LBM contamination. Although some of the factors like market disinfectant, wild bird contamination posses biological sense for the contamination but till now their role has not been studied in depth. So, this study has been conducted to identify the role of the previously reported risk factor and suspected risk factor for the occurrence of AIV.

2.7. Surveillance and Epidemiological Studies

Disease control can be improved when effective active or passive surveillance is in place which can detect the disease at very early stage. Live poultry and wild bird AI surveillance in Europe, North America and Hong Kong is very intensive, however, in Bangladesh the AI surveillance systems on domestic poultry in farms and LBM and wild birds are patchy and foreign fund-based (Negovetich et al., 2011b). LBM can maintain, amplify and disseminate the AIV to farms and also act as a source for human infection. So, LBM is very crucial to be considered for AI investigation in live poultry (ben Embarek et al., 2009).

Recently an active AI surveillance on LBM is going on through the UK funded BALZAC Project of Bangladesh with the active collaboration of the Royal Veterinary College, UK, Chittagong Veterinary and Animal Sciences University, Bangladesh and other national and international institutes and organizations. Also AI surveillance in both human and poultry at LBM is being conducted by International Center for Diarrheal Disease Research, Bangladesh. However, the presence of AIV in the environment of LBM was not well documented so far. Although an AI study at LBM in Chittagong and Dhaka Metropolitan cities has recently been published (Biswas et al., 2015), this study included only limited LBM to explore hygienic status

associated with the occurrence of AI. Therefore, the current study has been considered all LBMs under Chittagong Metropolitan area of Bangladesh to determine the association between hygienic practices and the occurrence of AI at LBMs.

2.8. Economic and Public Health Consequence

High prevalence of AI and its subtypes like HPAI H5NI at LBMs negatively impact on live bird trading at the markets because policy of market closure can be applied by the government. It happened earlier in LBMs of number countries across the world (Yu et al., 2014; Peiris et al., 2015). Although it may seem a simple matter to close wet markets for the general good of society but such closure would put thousands of vendors and workers in wet markets out of business which could produce economic loss as well as increase the price of the birds (Webster, 2004). The evidence of HPAI H5N1 produces destruction of the birds to prevent transmission of virus to human beings (Trampuz et al., 2004). Sometimes, the virus from LBMs can be spilled back to the farm by different means that produce destruction of the farm birds resulting high economic loss to the farmers (Trock et al., 1997; Trampuz et al., 2004; Kung et al., 2007a). If LBM environment constantly contaminated, AIV mutation and re-assortment can be happened. Therefore, a novel AI virus is likely to be emerged which can be a potential pandemic threat for the human population.

2.9. Diagnosis of Highly Pathogenic Avian Influenza Virus

Nucleic acid magnification for molecular identification have been identified as the most sensitive and speedy process for diagnosis of AIV (Pasick, 2008). In this respect Polymerase Chain Reaction (PCR) is the revolutionary molecular technique which can magnify a single or few copies of DNA to several-million-fold of copies. To use this technique for finding of AIV, a copy of DNA, complimentary (cDNA) to viral RNA, is synthesized (Dhumpa and Bang). PCR is more sensitive and time saving method than the traditional virus isolation method for identifying AIV (Fouchier et al., 2000).

A number of real time RT-PCR assays have been published to detect AIV RNA and different AIV genes from clinical and harvested samples (Arafa et al., 2012; Fereidouni et al., 2012; Heine et al., 2015). The sensitivity and specificity of real time

RT-PCR assays for environmental swab sample for Avian Influenza was reported to be 91.9% to 93.8% and 97.9% (Bulaga et al., 2003; Spackman et al., 2003).

So, the present study has planned to use Spackman assay (Spackman et al., 2003), Australian Animal Health Laboratory (AAHL) assay (Arafa et al., 2012; Fereidouni et al., 2012; Heine et al., 2015) because their sensitivity and specificity are quite high (around 95%).

2.10. Prevention and Control

Several studies has been reported that implementation of a rest day, improving market hygiene and not allowing live poultry to remain overnight in the market, discourage selling of wild-caught birds and provision of centralized slaughter house may reduce the LBM infection rate (ben Embarek et al., 2009). But before any intervention LBMs should scientifically be assessed for AIV status and the associated factors. Therefore, the present study has been conducted to determine the possible risk factors for the AIV contamination of LBMs in Chittagong, Bangladesh and accordingly recommend intervention measures.

The best indirect way to prevent the disease is to implement the biosecurity measures at the farm level. If biosecurity measures of a high standard are implemented and maintained at farm level, they create a firewall against penetration and perpetuation of the infection which ultimately protect the LBM from AIV infection (Capua and Marangon, 2006).

2.11. Conclusion

In conclusion LBM demography, prevalence of avian influenza along with risk factors, economic consequence, zoonotic significance, diagnostic approach along with prevention and control strategy in response to LBMs have been discussed and assessed the justifications to conduct the current investigation on the effect of hygienic status and occurrence of AI in LBMs under Chittagong metropolitan.

Chapter-III: Materials and Methods

3.1. Description of Study Site

Live Bird Markets (LBMs) of Chittagong metropolitan city, Bangladesh were chosen as study sites which is located 22°22'N and 91°48'E, and is 29 m up from the sea level. The metropolitan city is situated in the tropical zone and characterized by annual average range temperature of 13°C to 32°C, rain fall of 5.6mm to 727.0 mm and humidity of 70 to 85% (Anon, 2016a).

Chittagong coast city has very distinctive topography and a wide range of land types. However, the city lands are under continuous pressure due to rapid increase of population with growing demand for food. In order to meet the demand for animal protein peoples largely depend on poultry meat and eggs that can easily buy from city LBMs.

Global Positioning System coordinate data from each LBM and individual stall has been collected using Garmin eTsrex 10 machine in degree, minute and second format. Then the data were converted into decimal format and entered into a digitized map of Bangladesh. A geographic information system programme (ArcGIS-ArcMap version 10.2; Environmental System Research Institute, Redlands, CA, USA) was used to prepare a map showing the spatial distribution of the LBM and individual stalls under the LBM (Figure 1).

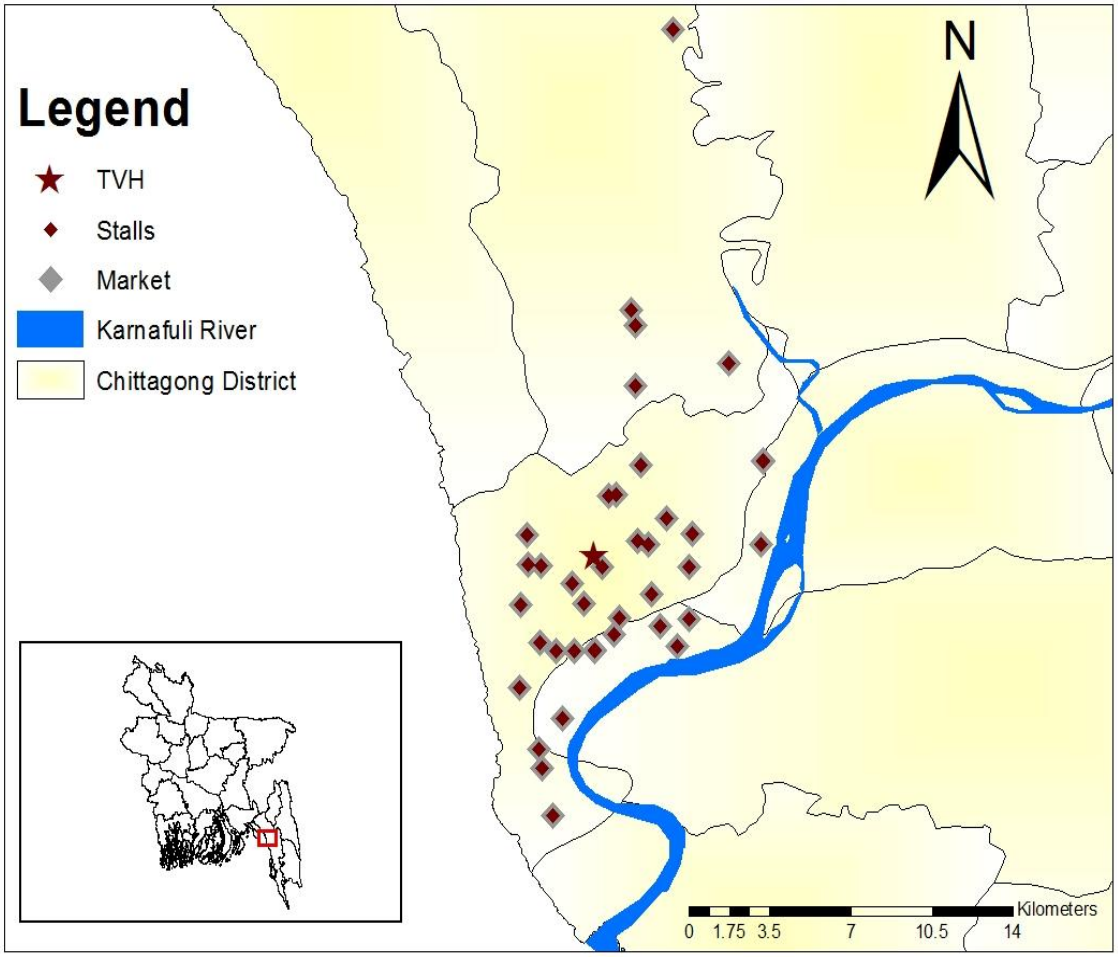

Figure 1: Location of Live Bird Markets of Chittagong Metropolitan City (*as coordinates of some of the markets are same, all 40 LBMs are not visualized individually)*

3.2. Live Bird Markets and Poultry Stalls

Both backyard and commercial poultry farming systems are being practiced in Chittagong like other parts of Bangladesh. Different poultry species (duck, chicken, pigeon, quail etc.) are playing important role as animal protein source for human consumption. Trading of live poultry widely occurs in different LBMs throughout Bangladesh. LBM is defined as an area where traders sell live poultry. "Live poultry" means finished birds which are intended to be slaughtered and eaten by the end user (Fournié et al., 2012a). Chittagong metropolitan city has 40 LBM with a 2-44 poultry stalls per market (Data unpublished, BALZAC Project, 2015). Some markets have both retail and whole sale poultry stalls. Most of the LBMs of Chittagong metropolitan city is poorly structured and poultry stalls in LBMs remain below

standard in terms of space for keeping birds, space for slaughtering, cleaning etc. Due to the shortage of space poultry of different species are kept together. The slaughtering of poultry is often performed within the stalls even though dedicated space for poultry slaughtering are seen in very limited LBMs. Manual or automated dressing of poultry birds are performed within stalls. In contrast, only few well structured LBMs with good facilities exists in Chittagong metro such as Hazi Abdul Ali Archid LBM and Shersha LBM.

Poultry of different species are entered into Chittagong metro city by vendors or businessman from Chittagong district and its surrounding districts (Cox's Bazar, Rangamati etc.) as well as districts of other parts of the country such as Pabna, Mymensingh, Kushtia and Sirajganj, Sunamganj and Sylhet (Hoque et al., 2014). This was another reason on why the present study was conducted in Chittagong Metropolitan LBMs.

3.3. Study Design, Sample Size and Sampling

A complete list of LBMs belonging to Chittagong Metropolitan City has been developed through the UK funded BALZAC Project being run its activities in Bangladesh with the active collaboration of the Royal Veterinary College, UK, Chittagong Veterinary and Animal Sciences University, Bangladesh and other national and international institutes and organizations. This list consists of 40 LBMs with a total of 398 poultry stalls. A range of 1-6 different poultry species (broiler, layer, indigenous, duck, quail, pigeon etc.) are reported to be traded in the LBMs. A range of 37 to 445 birds per LBM are reported to be sold out daily. All these 40 LBMs were included for the present cross-sectional study on avian influenza.

To investigate poultry stall level AIV prevalence, a total of 287 poultry stalls were required assuming 12% expected AI prevalence (Sukanta Chowdhury, ICDDR'B, Personal communication, 2016), ±1% precision, 95% confidence interval and 1% design effect.

All stalls (100%) were selected if a LBM had up to 10 stalls, whereas 50% stalls were selected when there were more than 10 stalls. Accordingly 290 poultry stalls were recruited which represented 72.9% of total poultry stalls under the LBMs of Chittagong Metro.

3.4. Collection, Preservation and Transportation of Field Sample

A pooled environmental swab sample (2-9 items per pool) per poultry stall was collected. The items of a pool consisted of poultry dropping, market floor, cage, feed, drain, slaughtering site, blood, offal's and waste bin (waste disposal area). Swab samples were collected by the investigator wearing the proper personnel protective equipment. Individual sample item was collected using sterile cotton swab stick (Sterile cotton swab, model: PW005 by HiMedia Laboratories) and then placed in 15 mL sterile Falcon tube (BD Biosciences, San Jose, CA) containing 5 ml viral transport media (VTM). The VTM was prepared according to recipe described by (WHO, 2006).

Falcon tubes with samples were given unique identification numbers, placed in insulated ice-box and transferred immediately to Poultry Research Training Centre (PRTC) laboratory, Chittagong Veterinary and Animal Sciences University. At the PRTC laboratory 2 aliquots per sample with a volume of 2 mL each were made. One aliquot was stored in -80^0C at PRTC Laboratory and another aliquot was forwarded to the National Reference Laboratory for avian influenza evaluation at Bangladesh Livestock Research Institute (BLRI). Samples were transported from PRTC to BLRI Laboratory and stored at -80^0C until analysis being performed.

3.5. Epidemiological Data Collection

A questionnaire was developed and reviewed by independent reviewer and pretested. Questionnaire was administered to either stall owner or employee to collect data by face to face interview. Data were also recorded by physical observation. Before the start of interview the participant vendors' consent was taken explaining the objectives and outcome of the study.

The questionnaire was structured in conformity with the study objectives under the three major headings *(the full questionnaire has been given in Appendix-A)*

- **Identity checklist:** This list consisted of market name, stall (shop) name and registration number, market GIS coordinate, name of the interviewer and interviewee.

- **Participant's demography:** The demography included profession, education, number of employee and types of poultry business.
- **Market structure, management and hygienic status based checklist:** The checklist items included total number of birds sold per day, location of the shop, poultry holding area, types of floor, housing of different poultry species, sources of poultry, activities carried out in the shop, management of leftover poultry at the end of daily selling, disposal of slaughter waste, wild birds contamination, separation of sick poultry from healthy poultry, management of sick poultry, outcome of slaughtered sick poultry, management of dead poultry, condition of waste bin, shop cleaning and disinfection and placement of poultry during disinfection/cleaning etc.

3.6. Laboratory Evaluation

3.6.1. Sample Preparation and RNA Extraction

All 290 pool swab samples were extracted as per the manufacturer protocol (RNeasy® Mini Handbook, 4th edition, April 2006 by Qiagen). In brief 300 µL samples were taken into an Eppendorf tube and then 400 µl RLT buffer was added. After vortex the mixture was left on the bench for 15 minutes. Then 700 µL of 70% ethanol was added to make the mixture up to 1400 µL. Afterwards 700 µL was transferred to spin column and centrifuged @ 13000 rpm for 1 minute. After pouring off the liquid portion 700 µL RW1 was added and again centrifuged @ 13000 rpm for 1 minute. Then spin column was transferred into a new collection tube followed by adding 500 µL RPE and centrifuged @13000 rpm for 1 minute. Again 500 µL RPE was added and centrifuged @13000 for 2 minute. The spin column was then transferred to a new Eppendorf tube. A volume of 50 µL RNAase free water was added at the middle of the column and centrifuged @13000 rpm for 30 second. After discarding of the spin column the Eppendorf tube with RNA extract was labeled and stored at -20^{0}C until being used for further molecular testing.

3.6.2. Real time Reverse Transcription Polymerase Chain Reaction

The published real time reverse transcription polymerase chain reaction protocols were used to evaluate avian influenza and its subtypes of H5, H7 and H9 (Arafa et al., 2012; Fereidouni et al., 2012; Heine et al., 2015). Molecular detection of AIV RNA

was performed using one step real time reverse transcriptase polymerase chain reaction (rRT-PCR) directed at the Matrix gene described by Heine et al. (2015). Any test sample that was reactive to the assay with a threshold value of ≤35 was considered as reactive for AIV RNA. Avian influenza reactive samples were further undergone for H5, H7 and H9 sub-typing evaluation using the rRT-PCR protocol described earlier (Arafa et al., 2012; Heine et al., 2015).

QIAGEN rRT-PCR kit was used for detection of Matrix gene, whereas AgPath rRT-PCR kit was used for detection of subtypes (H5, H7 and H9). **Stratagene Mx3005P QPCR** *(Aligent Technologies)* machine was used for testing samples.

The components of master mix of each assay along with the thermal profiles have been given in Table 3.1. The sequences of primers and probes used for the assays have been presented in Table 3.2.

Table 3. 1: Preparation of master mixtures with thermal profiles of the assays of real time reverse transcriptase polymerase chain reaction

Category	Item	Amount (µL)			
		M gene (Heine et al., 2015)	**H5 (Heine et al., 2015)**	**H7 (Fereidouni et al., 2012)**	**H9 (Arafa et al., 2012)**
Composition of master mixture	RNAase free water	14.10	0.124	2.25	2.0
	5X Buffer	5.0			
	dNTPs	1.0			
	$MgCl_2$	1.25			
	Forward primer	0.20		2.0	1.0
	Reverse primer	0.20		2.0	1.0
	Probe	0.25		0.25	1.0
	2X RT-Buffer mix		12.5	12.5	14.0
	Polymerase enzyme	1.0	1.0	1.0	1.0
	Duplex primer and probe mix		6.376		
	RNA template	5	5	5	5
	Total amount	28	25	25	25
Thermal profile	Reverse transcription	45°C for 10 minutes	50°C for 30 minutes	50°C for 30 minutes	50°C for 30 minutes
	Initial denaturation	95 °C for 10 minutes	95°C for 15 minutes	95°C for 15 minutes	95°C for 15 minutes
	No. of cycles	45 cycles	40 cycles	40 cycles	40 cycles
	Denaturation	94°C for 10 seconds	95°C for 15seconds	95°C for 15 seconds	95°C for 15 seconds
	Annealing	55 °C for 1 minutes	60 °C for 1 minutes	60 °C for 1 minutes	60 °C for 1 minutes
	Extension	60°C for 30 seconds	72°C for 10 seconds	72°C for 10 seconds	72°C for 10 seconds

Table 3. 2: Sequences of primers and probes used for detection of AIV M gene and H genes (H5/H7/H9)

Assay	Type	Name	Sequence (5´-3´)	References
Avian influenza matrix gene	Forward primer	IVA D161M	AGA TGA GYC TTC TAA CCG AGG TCG-900nM	(Heine et al., 2015)
	Reverse primers	IVA D162M1	TGC AAA AAC ATC YTC AAG TCT CTG-225nM	
		IVA D162M2	TGC AAA CAC ATC YTC AAG TCT CTG-225nM	
		IVA D162M3	TGC AAA GAC ATC YTC AAG TCT CTG-225nM	
		IVA D162M4	TGC AAA TAC ATC YTC AAG TCT CTG-225nM	
	Probe	IVA Ma	TCA GGC CCC CTC AAA GCC GA-250nM	
H5 subtype	Forward primers	IVA D148H5	AAA CAG AGA GGA AAT AAG TGG AGT AAA ATT-675nM	(Heine et al., 2015)
		IVA D204f	ATG GCT CCT CGG RAA CCC-675nM	
	Reverse primers	IVA D149H5	AAA GAT AGA CCA GCT ACC ATG ATT GC-675nM	
		IVA D205r	TTY TCC ACT ATG TAA GAC CAT TCC G-675nM	
	Probe	IVA H5a	TCA ACA GTG GCG AGT TCC CTA GCA-300nM	
		IVA D215P	ATG TGT GAC GAA TTC MT-300nM	
H7 subtype	Forward primer	H7 F	AYA GAA TAC AGA TWG ACC CAG T-800nM	(Fereidouni et al., 2012)
	Reverse primer	H7 R	TAG TGC ACY GCA TGT TTC CA-800nM	
	Probe	H7 Probe	TGG TTT AGC TTC GGG GCA TCA TG-100nM	
H9 subtype	Forward primer	H9 F	ATG GGG TTT GCT GCC	(Arafa et al., 2012)
	Reverse primer	H9 R	TTA TAT ACA AAT GTT GCA YCT G	
	Probe	H9 Probe	FAM-5' TTC TGG GCC ATG TCC AAT GG 3'-TAMARA	

3.7. Statistical Evaluation

Field and laboratory data were stored in Microsoft Excel 2007 spread sheet. Data were cleaned, coded and checked for integrity in MS Excel 2007 before exporting to STATA-IC-13 *(StataCrop, 4905, Lakeway Drive, College station, Texas 77845, USA)* for performing epidemiological analysis.

3.7.1. Descriptive Analysis

Descriptive statistics (frequency number and percentages) were calculated to express socioeconomic status of LBM stall vendors and stall hygienic status. Stall hygienic score was calculated based on five different factors: 1) Stall adjacent to other poultry shop (less than 20 feet) (Yes/No), 2) Separate housing for different species (Yes/No), 3) Wild birds contamination (Yes/No), 4) Separation of sick birds (Yes/No) and 5) Regular cleaning and disinfection of the stall (Yes/No). The mean hygienic score of stall under individual LBM represented the overall hygienic score of the subsequent LBM.

3.7.2. Univariate Analysis

Prevalence of avian influenza followed by different subtype was calculated based on different categories of bird species at stall level along with hygienic score at LBM level. The univariate Fishers exact test was used to compare AIV RNA screening results between and among the selected factors including dead bird disposal, separation of sick poultry and the stalls adjacent (within 20 feet) to the other stalls. The AIV subtype detection rate (H5 and H9) in respect to hygienic score at LBM level from the same sample was compared by using McNemar test.

3.7.3. Risk Factor Analysis

3.7.3.1. Univariate Analysis

Factors of selling of duck, access of wild birds to the stall, bird holding area and hygienic score were initially assessed by Fishers exact test to identify univariate association between the prevalence of AIV RNA and the selected aforementioned factors. The level of significance was set at $p \leq 0.05$.

3.7.3.2. Multivariate Analysis

Factors determined as significant ($p \leq 0.05$) by univariate Fisher's exact test were forwarded to multivariate analysis. To know the characteristics of the dataset first of all an ordinary logistic regression model ignoring clustering of stall was fitted. Then the same model was fitted asking for robust standard error. The calculated difference between likelihood-based (model-based) standard error and robust (residual-based) standard error represented the dataset as clustered where the market id was the cluster variable. Based on this context the Random Effect model was tried to be fitted for the data set. But the LRT (Likelihood Ratio Test) did not satisfy the model due to greater quadrature points (>0.01) at the 8^{th} number cut-point. So, alternative Generalized Estimating Equation model was performed considering the significant variables at 5% level of significance. A backward-elimination procedure was used by fitting the full model and reducing the model based on significance of the variables one by one. The confounding was checked out by adding or removing a variable from the model and the co-linearity has been checked out using Chi-square test between independent variables. If a variable could not be fitted due to presence of empty cell then that variable was excluded from the model. Variables that were significant ($p \leq 0.05$) based on Wald test were considered as risk factors for the Avian Influenza. The results were expressed as Odds Ratio (OR), Standard Error (SE) with 95% confidence interval.

Chapter IV: Results

4.1. Descriptive Analysis

4.1.1. Live Bird Poultry Stall Demographics

Fifty two percent poultry stalls were run by owners (n=151), whereas 48% stalls were run by workers (n=139). The educational qualification of owner was mostly class I- IX (57.6%) followed by SSC (16.6%), HSC (5.9%) and B.com (1.3%). Around 19% owners were illiterate. The educational qualification of worker was mostly class I-IX (79.9%) followed by SSC (4.3%), HSC (2.9%) and B.com (0.7%). Around 12.0% of workers were illiterate. The type of business was predominantly retail (75.5%) followed by both retail and wholesale (24.5%). Around 45% stalls had two workers; 26.5% stalls had three workers; 10% had four workers and 9.7% had more than four workers (Table 4.1).

Table 4.1: Socioeconomic status of live bird markets poultry stall vendors in Chittagong Metropolitan City, Bangladesh (N=40 markets, n=290 vendors)

Factor	Category	Frequency number	%
Profession	Owner	151	52.1
	Worker	139	47.9
Education (Owner)	Illiterate	28	18.5
	Class I-IV	52	34.4
	Class V-IX	35	23.2
	SSC	25	16.6
	HSC	9	5.9
	B.Com	2	1.3
Education (Worker)	Illiterate	17	12.2

	Class I-IV	49	35.3
	Class V-IX	62	44.6
	SSC	6	4.3
	HSC	4	2.9
	B.Com	1	0.7
Business type	Retail	219	75.5
	Both retail and wholesale)	71	24.5
Number of employees	1	24	8.3
	2	129	44.5
	3	77	26.5
	4	29	10.0
	>4	28	9.7
	n/a*	3	1.0

*n/a = Interviewee chose to withhold information; SSC: Secondary School Certificate; HSC: Higher Secondary Certificate; B.Com: Bachelor of Commerce.

4.1.2. Hygienic Status of Poultry Stalls of Live Bird Markets

Seventy three percent poultry stalls were closely located to the other stalls (within 20 square feet area). About 96% stalls had separate spaces for keeping bird species separately. Birds were mostly kept in cage of stalls (41.7%) followed by floor (29.4%) and both cage and floor (28.9%). Floor construction of the stalls were concrete (36.9%), mud (29.7%) and both concrete and mud (33.4%). Stalls were received birds from multiple sources of which 50.0% were supplied by wholesale markets; 46.2% were supplied by farms through middlemen; 0.3% were directly supplied by commercial farms and 3.5% were supplied by both farms through middlemen and commercial farms. Around 80% stalls did not have any space quarantine for keeping sick birds. Resident wild bird such as crow had access to 48.3% stalls (Table 4.2).

For cleaning and sanitation, 75.9% stall vendors responded that they cleaned or washed their poultry stalls daily of which 54.5% used only water and 45.5% used disinfectant or detergent. Among all disinfectants bleach powder was most commonly used (72.7%).

In addition to bird selling, slaughtering (90.0%), manual dressing (81.4%) and machine dressing (40.0%) had been practiced in the stalls. Almost 100% vendors kept their left over birds in the stall (either in the cage or floor) for just immediate next day selling. Solid wastes were generally disposed into dustbin (76.6%), drum (1.4%), drain (7.9%), water bodies like pond, river etc. (2.7%) and beside the highway or another open space (2.8%). Sometimes, the vendors sold wastes as a fish feed (1.7%). Liquid wastes were mostly disposed in the dustbin (73.1%), common drain (7.6%), water bodies (pond, river etc) (3.4%) and beside the highway or any other open space (1.0). Mean hygienic score of stalls mostly lied between 2 and 3 (70.0%) (Table 4.2).

Table 4.2: Poultry stalls hygienic status of live bird markets (N=40 markets, n=290 vendors)

Factor	Category	Frequency number	%
Location to other LBM stalls	Adjacent (within 20 feet)	212	73.1
	Not adjacent	78	26.9
Separate housing for each species	Yes	277	95.5
	No	13	4.5
Poultry holding area	Cage	118	40.1
	Floor	124	42.8
	Both	48	16.5
Floor construction	Smooth (Concrete)	107	36.9
	Rough (Mud)	86	29.7
	Mixed (Concrete and Mud)	97	33.4
Poultry source	Wholesale	145	50
	Commercial farms	134	46.2

	through middlemen		
	Directly from commercial farm	1	0.3
	Multiple sources (both middlemen and commercial farms)	10	3.5
Resident Wild bird contamination *(eg: crow)*	Yes	140	48.3
	No	150	51.7
Sick poultry separation	Yes	50	17.2
	No	240	82.8
Clean frequency (dry or moist cleaning)	<1x per day	34	11.7
	1x per day	220	75.9
	2x per day	23	7.9
	>2x per day	13	4.5
Stall disinfection	Yes	132	45.5
	No	158	54.5
Disinfectant type	Bleach powder	99	74.4
	Others *(Timsen, surf excel, wheel powder, soap etc.)*	34	25.6
Water source	Supply	249	85.9
	Store	37	12.8
	Mixed (supply and store water)	2	0.7
	No use of water	2	0.6
Services offered at stalls	Selling	290	100
	Slaughter	261	90
	Machine dressing	116	40
	Manual dressing	236	81.4

	Evisceration	173	59.7
	Cutting meat	29	10
Left over bird use	Just immediate next day sale	289	99.7
	No left over	1	0.3
Solid waste disposal	Dust bin	222	76.6
	Drum	4	1.4
	Drain	23	7.9
	Sold for fish feed	8	2.8
	Water bodies (pond, river)	8	2.7
	Open space (beside highway, unused land etc)	5	1.7
	Not specific site for disposal	20	6.9
Liquid waste disposal	Dust bin	212	73.1
	Drum	10	3.5
	Drain	22	7.6
	Sold for fish feed	2	0.7
	Water stream (pond, river etc)	10	3.4
	Open space (beside highway, unused land etc)	3	1
	No specific site for disposal	31	10.7
Overall hygienic score (shop)	0	1	0.4
	1	70	24.1
	2	59	20.3
	3	148	51
	4	9	3.1
	5	3	1

4.1.3. Frequency of Avian Influenza and the Selective Subtype According to Categories of Bird Species

Around 28% stalls traded one poultry species (either broiler chicken or deshi indigenous chicken) and 72% traded 2 or more than 2 species (Table 4.3).

The prevalence of AI in the stalls with single species ranged from 22.5 to 50%, whereas the prevalence of AI in stalls with 2 or more species ranged from 5.2 to 34.5% (Table 4.3).

Regardless of single or multiple poultry species traded both H5 and H9 were detected across the stalls. However, one stall had a concurrent infection of both H5 and H9 (Table 4.3).

Table 4. 3: Distribution of Avian influenza and its subtype specific prevalence in stalls of live bird markets (by different classes of poultry species traded)

Species	No. of Stalls (%)	AIV (%)	H5 (%)	H7 (%)	H9 (%)	Un-typed (%)
BC only	80 (27.6)	18 (22.5)	2 (2.5)	---	1 (1.3)	16 (20.)
BC and LC or DC	106 (36.6)	14 (13.2)	2 (1.9)	---	3 (2.8)	10 (9.4)
BC, LC and DC	57 (19.7)	3 (5.2)	1 (1.8)	---	1 (1.8)	1 (1.8)
BC, DC, LC and P/D	28 (9.6)	3 (10.7)	---	---	---	3 (10.7)
BC, LC, DC, D and P /Q	58 (20.0)	20 (34.5)	3 (5.2)	---	3 (5.2)	14 (24.1)
Only DC	2 (0.7)	1 (50.0)	---	---	1 (50)	---

BC: Broiler Chicken; LC: Layer Chicken; DC: Deshi (indigenous) Chicken; D: Duck; P: Pigeon; Q: Quail.

4.1.4. Hygienic Score and Individual Live Bird Market Infection

At least a LBM with one or more AI positive stall (s) was considered as AI positive market. Also at least a LBM with one or more AIV H5 and or H9 positive was defined as H5 or H9 positive market. At LBM level the prevalence of AI significantly varied by mean hygienic score (p=0.01) (Table 4.4). The LBMs with the hygienic score of >3 had zero AI case. However, the prevalence of AI was significantly greater in LBMs with the score of ≤2.4 (81.3%) than in LBMs with the score of 2.5-3 (18.7%) (p=0.002).

No difference was observed for the prevalence of H5 between the LBMs with ≤2.4 score (50.0%) and LBMs with ≥2.4 score (50.0%). All H9 cases were detected in LBMs with ≤2.5 score. No difference was observed between the LBM prevalence of H5 and H9 (p=1.0).

Table 4.4: Association between mean hygienic score of LBMs and the prevalence of AIV/H5/H7/H9 and other subtypes

Hygienic score	No. of LBMs	AIV (%)	H5 (%)	H7 (%)	H9 (%)	Un-typed (%)
>3	3	0	0	0	0	0
2.5-≤3	16	3 (18.7)	3 (50.0)	0	0	1 (7.1)
Min-2.4	21	13 (81.3)	3 (50.0)	0	5 (100)	13 (92.9)
Total	40	16 (40.0)	6 (15.0)	0	5 (12.5)	14 (35.0)

LBM: Live Bird Market

4.1.5. Prevalence of Avian Influenza and the Selective Subtypes (Both Live Bird Market and Stall Level)

At LBM level the overall avian influenza (AI) prevalence was 40% (95% CI: 20-60%; N=40) of which the prevalence of H5 was 15.0% (95% CI: 6-30%), H7 was zero; H9 was 12.5% (95% CI: 4-30%) and un-typed was 35.0% (95% CI: 20-50%). On the contrary, no significant difference was evidenced between the prevalence of H5 and H9 in different LBMs (p=1.0).

At stall level the overall AI prevalence was 20.3% (95% CI: 10-30%, N=290) of which the prevalence of H5, H7 and H9 was 2.8% (95% CI: 1-5%), 0% and 3.1% (95% CI: 1-6%), respectively. The prevalence of AIV un-typed was 15.2% (95% CI: 10-19%) *(the detailed information has been given in Appendix-B).*

4.2. Risk Factor Analysis for Live Bird Market Contamination by Avian Influenza

4.2.1. Univariate Analysis

The prevalence of AI varied significantly by selling of different species, dead bird disposal, and access of wild bird to the stalls, bird holding area and overall mean hygienic score of the stalls ($p \leq 0.05$). The stalls traded duck with other bird species had significantly higher AI prevalence (34.8%) than the stalls traded only non duck species (p=0.001). The stalls disposed dead birds in dustbin and drain had significantly higher AI prevalence (21.9%) than the stalls disposed dead birds in water bodies like pond, river etc (0%) (p=0.05). However, the stalls with the access of crow had significantly higher AI prevalence (33.6%) than the stalls without access (8.0%) ($p<0.001$). Again stalls kept birds on floor system had significantly higher AI prevalence (29.7%) than the stalls kept birds in cage system (12.9%) (p=0.001). The stalls with lower hygienic score (0-2.9) had significantly higher prevalence of AI (34.6%) than the stalls with higher hygienic score (8.8%) ($p<0.001$).

Table 4.5: Univariate association between factors and the prevalence of avian influenza in stalls under different live bird markets (N=290)

Factor	Category	N	Avian Influenza		p- (Fisher's exact test)
			Positive (%)	Negative	
Selling of Species	Only chicken	200	34 (17.0)	166	0.002
	Chicken and non-duck species	21	1 (4.8)	20	
	Duck with other species (eg; Chicken, Quail, Pigeon etc.)	69	24 (34.8)	45	
Selling of species	Chicken and non duck species	221	35(15.8)	186	0.001
	Duck with other species	69	24(34.8)	45	
Dead bird disposal	Drain and Dustbin	269	59 (21.9)	210	0.05
	Sold out as fish feed	12	0	12	
	Water bodies (pond, river etc)	9	0	9	
Access of wild birds to the stall	Yes	144	47 (33.6)	97	0.000
	No	146	12 (8.0)	134	
Bird holding area	Cage	162	21 (12.9)	141	0.001
	Floor	128	38 (29.7)	90	
Separation of sick poultry	Yes	50	7 (14.0)	43	0.252
	No	240	52 (21.7)	188	
Adjacent (within 20 feet) to the other stalls	Yes	212	47 (22.1)	212	0.250
	No	78	12 (15.3)	78	
Mean hygienic Score of stalls	0-2.9	130	45 (34.6)	85	0.000
	3.0-5.0	160	14 (8.8)	146	

4.2.2. Generalized Estimating Equation

Factors determined as significant ($p \leq 0.05$) by univariate Fisher's exact test were forwarded to multivariate Generalized Estimating Equation (GEE) analysis in order to assess the adjusted population averaged effects on the prevalence of AI. After adjustment of the effect of factors each other, selling of species, bird holding area, wild bird access and mean hygienic score were identified as potential risk factors for the occurrence of AI in poultry stalls but due to co-linearity wild bird access was removed from the model.

Table 4. 6: Outputs of Generalized Estimating Equation model

Factors	Category	OR	95% CI	p-value
Selling of species	Chicken and non-duck species	1.0		
	Duck with other (eg; Chicken, Quail, Pigeon etc.)	2.5	1.5-4.1	<0.001
Bird holding area	Cage	1.0		
	Floor	1.9	1.1-3.4	0.03
Hygienic score	≥3	1.0		
	<3	3.1	1.7-5.6	0.000

Stalls traded duck with other poultry species were 2.5 times more likely to have occurred AI than the stalls traded only non duck species of poultry (95% CI: 1.5-4.1; $p<0.001$). The prevalence of AI was 1.9 times higher among those stalls where birds kept on floor than among those stalls where birds kept in cage (95% CI: 1.1-3.4; $p=0.03$). However, stalls with ≤3 hygienic score were 3.1 times more likely to have AI than stalls with ≥3 hygienic score (95% CI: 1.7-5.6; $p=0.00$).

Chapter V: Discussion

Epidemiological surveillance and studies of avian influenza have so far predominantly focused on commercial and backyard poultry farms in Bangladesh (Biswas et al., 2008a; Biswas et al., 2009). However, many earlier studies in different countries have suggested that Live Bird Markets (LBMs) have potential role on the avian influenza epidemiology in terms of introduction of Avian Influenza (AI) to LBMs through a complex live bird trading transaction chain, amplification and spreading of avian influenza viruses (Van Kerkhove et al., 2009). But very limited and well structured avian influenza published studies have previously been performed on LBMs in Bangladesh (Biswas et al., 2015) (Data unpublished, Sukanta Chowdhury, ICDDR'B, Personal communication, 2016). Therefore, the present cross sectional study was conducted on all LBMs belonging to Chittagong Metropolitan city, Bangladesh in order to assess avian influenza status and the associated factors. This section of the thesis has discussed important findings of the current study and their implications along with limitations and, conclusion and recommendations.

5.1. Live Bird Market Demography and Hygienic Status of Poultry Stalls

Regardless of types most of the stall vendors had I-IX level of education (57.6-79.9%), with a 12-19% illiterate in the present study. This result suggests that vendors can read Bengali literature easily; and therefore it would be easy and efficient to educate stall vendors on LBM hygiene through providing simple leaflet and manual with the aim of reducing zoonotic disease risk.

Three quarters of poultry stalls were retail type and the rest was both retail and wholesale types which reflect the other big city markets of the country, are also supported by the study conducted in Indonesia (Indriani et al., 2010b). As business pattern varies between retail and wholesale stalls, therefore disease risk for introduction, amplification and dissemination of organisms will vary between the stall types. Hence, it would be much safer to reduce zoonotic disease risk if any LBM has only single type poultry stall and business.

The present study identified that the stalls' floor was constructed with mud (29.7%) and both concrete and mud (33.4%). Muddy floor is tough to clean and disinfect and sometimes it can hold the water which ultimately produces unhygienic condition of the stalls and floor (either mud or concrete) get contaminated easily with infectious agents than cage system of keeping poultry (Indriani et al., 2010b).

Most of the poultry stalls were located closely (73%) in this study and therefore any infectious agent can easily be transmitted from one stall to another.

In the present study stalls received birds from multiple sources (wholesale markets, middlemen, commercial farms etc.), and birds are kept them together during transportation, like other countries such as Indonesia (Indriani et al., 2010b). The actual source of bird is either patio or little scale business ranches, with a low level of biosecurity that has been reported in many countries including Bangladesh, Vietnam and Indonesia (Fournié et al., 2012b). An earlier study reported that in Bangladesh farmers facing outbreak tried to sell out the apparently healthy or affected poultry to minimize their economic loss (Fournié et al., 2012b). However, it is very difficult to identify the actual source of infected birds in the stall when the birds are received from multiple sources.

Around 80% stalls did not have any quarantine space for keeping sick birds in the current study. So, healthy birds in stalls might easily be contaminated by sick birds. Poultry stalls having mixed of different species from different sources in this study also create an environment for infectious pathogens to transmit from one bird species to another bird species and also from birds to human (Mondal et al., 2012). So space provisioned for sick birds should be introduced.

Wild bird such as crow had access to 48.3% stalls in the present study and access of wild birds to the feeding and watering hotspots for poultry may likewise permit round about transmission. Again wild birds could transmit the contamination to local flying creatures and assume a part in the spread of infection between farms (Fournié et al., 2012b). So biosecurity measures should be taken to the individual stalls of LBM.

More than 50% stalls used only water for cleaning the stalls in the current study. Whereas the avian influenza virus can be inactivated using detergent treatment like calcium hypochlorite (750 ppm), sodium hypochlorite (750 ppm), powdered laundry detergent etc (Lombardi et al., 2008). So, the poultry market authority and stalls' vendors should be educated on how to prevent virus introduction, amplification and transmission using appropriate detergents for cleaning stalls.

In this study slaughtering of poultry was mostly performed within the stalls followed by predominantly manual dressing of poultry and these practices can lead to contaminate poultry carcasses and in turn transmit infectious pathogens to humans which are supported by earlier study conducted in Indonesia (Indriani et al., 2010b). So the structured and separate slaughter house within each LBM is highly desirable to prevent infection transmission in the chain of bird-bird-human.

Almost 100% stalls vendors kept their left over bird in the stall for the next day selling in the present study which could be a potential source for LBM contamination (Bulaga et al., 2003; Leung et al., 2012). Emptying of live bird in the market at night have been reported to produce lack of permissive host for the virus. So, the virus became unable to replicate (Peiris et al., 2015).

In the current study wastes were generally disposed into open dustbin, drain, water bodies and beside the highway or any open space that make the wild bird specially crow to attract the viscera for feed. Feeding of infected viscera may establish infection within the birds which may be further transmitting to the small and large scale poultry ranches.

5.2. Avian Influenza Prevalence and Associated Risk Factors

LBM level AIV prevalence of 40% in this study is supported by a study conducted in Indonesia (47%) (Indriani et al., 2010b), but this prevalence is higher than the LBM AI prevalence of 12.4% and 19.5% reported, respectively in Egypt and Hong Kong (Abdelwhab et al., 2010; Indriani et al., 2010b; Fournié et al., 2012b).

Stall level AI prevalence was 20.3% in this study which is higher than the study conducted previously in Chittagong (12.0%) and Khulna (10.0%) but lower than in

Dhaka (29%), Rajshahi (23.0%), Sylhet (50.0%), Barisal (25.0%) and Rangpur (50.0%) (Data unpublished, Sukanta Chowdhury, ICDDR'B, Personal communication, 2016).

The present study identified the selling of duck with other species in stalls as a risk factor (OR=2.5; 95% CI: 1.5-4.1) for the occurrence of AIV and this finding is agreed with an earlier study conducted by Zhou et al. (2015) in China (p=0.039) and Indriani et al. (2010b) in Indonesia.

The bird holding system (Floor/Cage) in the stall was also determined as a risk factor for the occurrence of AIV (OR=1.9; 95% CI: 1.1-3.4; Floor versus Cage) in this study. This finding corresponds to many previous studies where bird holding area identified as a risk factor as well as a Critical Control Point (CCP) for prevention of AIV transmission (Indriani et al., 2010b; Samaan et al., 2011). The higher odds of AIV in birds holding on floor system might be due to floor contamination as birds (healthy and sick) share the same bedding materials and utensils for feeding and watering which has also been reported by Martin et al. (2006).

The stall hygienic score was also identified as risk factor in this study. The odds of AIV in score of <3 was significantly higher (OR=3.1; 95% CI: 1.7-5.6). This appears a new findings as published literature identified LBM hygiene being associated with AIV (Webster, 2004; ben Embarek et al., 2009; Fournié et al., 2013).

5.3. Discussion on Sub-type Specific AI Prevalence (H5, H7 and H9)

Sub-type specific AI prevalence at stall level was low in this study (2.8% H5 and 3.1% H9). These findings are identical to other Bangladesh studies (4% H5 and 14% H9) (Data unpublished, Sukanta Chowdhury, ICDDR'B, Personal communication, 2016). This seems a new finding because many of the studies represented the sub-type specific prevalence at LBM level rather than stall level (Indriani et al., 2010a; Negovetich et al., 2011a).

On contrary sub-type specific AI prevalence at LBM level was higher (15.0% H5 and 12.5% H9) than the prevalence at stall level in the present study, however is lower than

Indonesia (50% H5) (Indriani et al., 2010a) and Bangladesh (Dhaka city 16.5% H9) but higher than in South Korea (7-8% H9) (Negovetich et al., 2011b).

The present study estimated zero prevalence for H7 at stall and market level which is well agreed with an earlier study performed in LBMs of many Asian countries (ben Embarek et al., 2009). This suggests that the H7 subtype is not circulating at LBM in Chittagong.

The prevalence of un-typed AI was quite high in this study (35.0%) which is an identical result of an earlier study in Bangladesh (Sukanta Chowdhury, ICDDR'B, Personal communication, 2016) suggesting other AI subtypes may be circulating in the LBM.

5.4. Limitations of the Current Study

An impediment of this study is that the perception of environmental contamination depended on a cross-sectional review in which LBMs were tested just once. Another impediment is a big fluctuation of swab items in a pool (2-9). This is due to the availability of the sampling sites and the willingness of the traders for allowing sample collection. There might be information bias aroused due to hiding information by the vendors despite motivating them to participate with the study willingly.

Chapter VI: Conclusions, Recommendations and Future Directions

Stall vendors had predominantly I-IX level of education. Three quarters of poultry stalls were retail type and most of the stalls were closed together. Around one third of stalls' floor was constructed by mud. Stalls received birds from multiple sources, had commonly no quarantine space for sick birds and had access to resident wild birds. More than 50% stalls used only water for cleaning the stalls. Each stall had unsold birds which stayed overnight in the stall for the next day selling.

Slaughtering of poultry was mostly performed within the stalls and wastes were generally disposed into open dustbin, drain, water bodies and beside the highway or other open space.

The prevalence of avian AIV at LBM and stall level was 40% and 20.3%, respectively. The subtype specific prevalence was 15.0% H5, 0% H7 and 12.5% H9 (LBM level) and 2.8% H5, 0% H7 and 3.1% H9 (Stall level).

Duck with other species in stalls, birds keeping on floor and poor hygienic score of stalls were determined as potential risk factors associated with the occurrence of AI prevalence. Therefore, needful technique to reduce the AI infection level at LBM along with public awareness through motivation and education should be sought in future. As an immediate suggestion supplying of leaflet written in Bengali on the risk factors identified in this study for AI threat could be able to rise up consciousness of the vendors. However I recommend for future studies is to watch steadiness of the infection over time in different environmental sites of LBMs by collection of swab samples maintaining homogeneity for every stalls. Again the author wants to provide the following directions for the future:

1. A longitudinal or repeated cross-sectional study should be conducted to assess the temporal pattern of AI in LBMs.
2. A cohort study or Intervention study covering wider study sites including rural and city LBM of Chittagong district should be considered.

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Appendix A: A Questionnaire on Epidemiological Assessment of Hygienic Conditions of Live Bird Markets on Avian Influenza in Chittagong Metropolitan City, Bangladesh

Objectives

1 To evaluate the Live Bird Market demography and hygienic status in Chittagong Metropolitan City

2 To estimate the prevalence of avian influenza virus and the selective subtypes of H5, H7 and H9 at stall and live bird market levels in Chittagong Metropolitan City

3 To determine potential risk factors associated with the occurrence avian influenza at stalls of live bird markets in Chittagong Metropolitan City

1. Identity Information

Market name:

Vendor/Shop name and number:

GPS code:

Market: _____(N) and _____ (E)

Shop: _____(N) and _____(E)

Interviewer's name:

Name of interviewee:

2. Socio-Economic Status

Profession: Stall owner/Poultry Worker

Education: Illiterate/Class I-V/Class V-IX/Secondary School Certificate (SSC)/Higher Secondary Certificate (HSC)/ More (specify).....

Number of employees:

Type of poultry business: Retail/Wholesale/Mixed

3. Market Structure, Management, and Hygienic Status

Location of stall: Adjacent to other poultry stall (within 20 feet)/Not adjacent to other poultry stall

Poultry holding area: Cage/Floor/Bamboo nest/Other (specify)

If floor, construction of floor: Smooth/Rough/Mixed/Other (specify)

Floor of stall (√)	Yes	No	Comments
Tiles			Full/partial
Concreted			Full/partial
Dirt			Full/partial
Others			

Are different species of poultry housed separately? Yes/No

Source of poultry: Wholesale market/Commercial farms/Backyard farms/Multiple sources (market and farm)/Middlemen/Other (specify)

What kinds of activities are carried out in your stall?

Activities	Broiler chicken	Layer chicken	Domestic chicken	Duck	Geese	Quail	Pigeon	Others
Selling								
Slaughtering								
Hot water								
Manual defeathering								
Evisceration								
Cutting meat								
Others								

Management of leftover poultry at the end of daily selling: Keeping for sale on next day/Supply to other stall/Supply to restaurant/Other (specify)

Does the stall have machine hot water dressing? Yes/No

If Yes, what percentage of poultry dress daily using machine?

Disposal of slaughter waste:

	Solid waste	Liquid waste
Through drain		
In drum		
In dust bin		
In the open space		
Other		
No disposal		

Do you see wild birds (i.e. crow) visit your stall: Yes (always)/Yes (sometimes)/No

Do you separate sick poultry from healthy poultry? Yes/No

Management of sick poultry: Slaughter for sale/Wait for recovery/Sell sick poultry/ Other (specify)

Outcome of slaughtered sick poultry: Sell to regular customer/Sell to regular restaurant/Slaughter for self-consumption/Slaughter for destruction/Other (specify)

Management of dead poultry: Disposed through drain/Disposed in drum/Disposed in dust bin/Disposed in open space/Other (specify)

Condition of waste bin (drum): Porous/Sealed

Stall cleaning:

Place/ Materials	Wet clean	Using clean water/ drain water/ reuse water	Dry clean	Sweeping/ using shovel/ brushing/ grooming	Daily basis/ weekly basis	How many times/day	How many times/ Week
Floor							
Work surface (bench/chopping board)							
Utensils							
Cages and other poultry holding area							
Others							

Stall disinfection: Yes/No

Place/ Materials	Daily basis/ weekly basis	How many times / day	How many times / week	Type of disinfectant (Virkon S /Timsen /Emsen/ bleaching powder/ detergent /soap/other.....)	Provided by (self/employee/ market committee /City Corporation/NGO /FAO/other.....)
Floor					
Work surface (bench/ chopping board)					
Utensils					
Cages and other poultry holding area					
Others					

Placement of poultry during disinfection/cleaning: Same cage/Different cage/Other (specify)

Environmental sample taken: Poultry droppings/Floor/Cage surface/Feed/Drain/ Slaughtering place/Blood/Offals/Waste disposal bin/Other (specify)

Water and others used for (√):	Cleaning waste	Dressed chicken	Hand Wash	Chicken drinking
Supply water				
Store water				
Recycling contaminated water				
Drain water				
No water supply				
Using towels, paper (others.....)				

Notes:

Interviewer Signature: _____Date: _____

Appendix B: Total number of stall with sampled number of stall along with Prevalence of AI, H5, H7 and H9 at individual LBM along with means hygienic score.

Market	Total no. of stalls	Sampled number of stalls	Mean hygienic score	Range of scores	AIV positive stalls (%)	95 % CI	H5 positive (%)	H7 positive (%)	H9 positive (%)	Un-typed (%)
Amanbazar	6	6	1.7	1-3	6 (100%)	50-100 *	0 (0%)	0 (0%)	1 (16.7%)	5 (83.3%)
Anandabazar-1	2	2	3.0	3	0 (0%)	0-80*	---	---	---	---
Anandabazar-2	12	6	3.0	3	0 (0%)	0-50*	---	---	---	---
Artillary bazar	6	6	1.2	1-2	6 (83.3%)	40-100	0 (0%)	0 (0%)	0 (0%)	6 (100%)
Bahaddarhat	22	11	2.3	1-4	5 (45.5%)	20-80	0 (0%)	0 (0%)	3 (60%)	2 (40%)
Balusara bazar	4	4	1.5	1-2	0 (0%)	0-60*	---	---	---	---
Bandortilla	18	9	2.6	1-3	1 (11.1%)	0.2-50	1 (100%)	0 (0%)	0 (0%)	---
Baropole	6	6	1.7	1-3	2 (33.3%)	4-80	0 (0%)	0 (0%)	0 (0%)	2 (100%)
Beparipara	8	8	1.9	1-3	0 (0%)	0-40	---	---	---	---
Bibirhat Kachabazar	6	6	3.0	3	0 (0%)	0-50*	---	---	---	---
Bolirhat	9	9	2.2	1-3	0 (0%)	0-30*	---	---	---	---
Botola bazar	4	4	3.3	2-5	0 (0%)	0-60*	---	---	---	---
Bow bazar	4	4	2.8	2-4	4 (100%)	40-100*	1 (25%)	0 (0%)	0 (0%)	3 (75%)
Boxirhat	6	6	2.7	2-4	0 (0%)	0-50*	---	---	---	---
CDA Karnafuli	18	9	2.6	1-3	0 (0%)	0-30*	---	---	---	---
Chittagong Export Processing Zone Market	5	5	1.4	1-3	4 (80.0%)	30-100	1 (25%)	0 (0%)	0 (0%)	3 (75%)
Chalkbazar	10	10	2.2	1-3	0 (0%)	0-30*	---	---	---	---
Chotopole	5	5	1.8	1-3	4 (80.0%)	30-100	---	---	---	4 (100%)
Chowdhuryhat	7	7	2.4	1-3	0 (0%)	0-40*	---	---	---	---
Dewanhat	4	4	2.5	1-3	0 (0%)	0-60*	---	---	---	---
Fatahabad	6	6	3.2	3-4	0 (0%)	0-50*	---	---	---	---

Firingi bazar	5	5	2.8	2-3	1 (20.0%)	5-70	1 (100%)	0 (0%)	0 (0%)	---
Foillatoli	11	6	1.7	1-3	0 (0%)	0-50*	---	---	---	---
Hazi Abdul Ali Archid	6	6	3.5	3-5	0 (0%)	0-50*	---	---	---	---
Jahutola kachabazar	14	7	2.0	1-3	2 (28.6%)	4-70	0 (0%)	0 (0%)	1 (50%)	1 (50%)
Kalamia bazar	5	5	1.8	1-3	0 (0%)	0-50*	---	---	---	---
Kamal bazar	5	5	3.0	3	0 (0%)	0-50*	---	---	---	---
Kaptai Raster matha	7	7	2.6	2-3	0 (0%)	0-40*	---	---	---	---
Karnafuli complex	42	21	2.4	1-3	3 (14.3%)	3-40	---	---	---	3 (100%)
Kazirdewri	9	9	2.0	0-3	8 (88.9%)	50-100	1 (12.5%)	0 (0%)	1 (12.5%)	7 (87.5%)
Komolmohazone	5	5	3.0	2-5	0 (0%)	0-50*	---	---	---	---
Kornelhat	7	7	2.7	1-3	0 (0%)	0-40*	---	---	---	---
Muradpur	5	5	2.8	2-8	0 (0%)	0-50*	---	---	---	---
Oxygen	9	9	2.3	1-3	5 (55.6%)	20-90	0 (0%)	0 (0%)	3 (60%)	2 (40%)
Pahartoli	20	10	2.3	1-3	0 (0%)	0- 30*	---	---	---	---
Riazuddin	48	24	2.2	1-4	4 (16.7%)	5-40	2 (50%)	0 (0%)	0 (0%)	2 (50%)
Sagorika	6	6	1.8	1-3	4 (66.7%)	20-90	**1** (25%**)**	0 (0%)	0 (0%)	3 (75%)
Shershah	5	5	3.0	2-4	0 (0%)	0- 50*	---	---	---	---
Steelmill	10	10	2.4	1-4	1 (10.0%)	0.2-40	---	---	---	1 (100%)
Yesinarhat	11	5	3.0	3	0 (0%)	0-50*	---	---	---	---
Total		**290**			**59 (20.3%)**		**8 (2.8%)**	**0**	**9 (3.1%)**	**44 (15.2%)**

CI: Confidence Interval; * use the following command: one-sided, 97.5% confidence interval had calculated for all positive and or all negative samples.

Brief Biography

Md. Abu Sayeed passed the Secondary School Certificate Examination (SSC) in 2004 obtaining GPA 5.00 and then Higher Secondary Certificate Examination (HSC) in 2006 obtaining GPA 5.00. Mr. Sayeed obtained his Doctor of Veterinary Medicine Degree in 2012 (held in 2014) from Chittagong Veterinary and Animal Sciences University (CVASU), Bangladesh. Now, he is a candidate for the degree of MS in Epidemiology under the Department of Medicine and Surgery, Faculty of Veterinary Medicine, CVASU. He has immense interest to work in Avian Influenza and live bird trading network.