SEROPREVALENCE OF LOW PATHOGENIC AVIAN INFLUENZA (H9) IN SONALI CHICKENS OF JOYPURHAT

A THESIS

BY

Md. Ziaur Rahman

Registration No.1405090

Session: 2014-15

Semester: Jan-June

MASTER OF SCIENCE (M.S.)

IN

PATHOLOGY

DEPARTMENT OF PATHOLOGY AND PARASITOLOGY

FACULTY OF VETERINARY AND ANIMAL SCIENCE

HAJEE MOHAMMAD DANESH SCIENCE AND TECHNOLOGY UNIVERSITY

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The Author

Hajee Mohammad Danesh Science and Technology University, Dinajpur.

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Dedicated to My Parents

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LIST OF ABBREVIATIONS AND ACRONYMS

AI	Avian influenza
AIV	Avian influenza viruses
BSA	Bovine serum albumin
cRNA	Complementary ribonucleic acid
EDTA	Ethylenediaminetetraacetic acid
FAO	Food and Agriculture Organization
GM	Growth medium
HA	Hemagglutinin
HPAI	High pathogenic avian influenza
HPAIV	High pathogenic avian influenza virus
ICDDR'B	International Centre for Diarrhoeal Diseases Research Bangladesh
LPAI	Low pathogenic avian influenza
LPAIV	Low pathogenic avian influenza virus
M	Matrix
mRNA	Messenger ribonucleic acid
NA	Neuraminidase
NEP	Nuclear export protein
NHEB	Normal human bronchial epithelial
NP	Nucleoprotein
NS	Non-structural
NLS	Nuclear localization signal
OD	Optical density
OIE	World Organization for Animal Health (Office International des Episooties)
PB1	Polymerase basic 1

PB2	Polymerase basic 2
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PKR	dsRNA-dependent protein kinase
qRT-PCR	Quantitative real time RT-PCR
RBCs	Red blood cells
RNA	Ribonucleic acid
vRNA	Viral ribonucleic acid
VGM	Virus growth medium
WHO	World Health Organization
%	Percent
&	And
aa	Amino acid

ABSTRACT

The present study was designed to know the sero-prevalence status of H9 antibodies in sonali chickens at Joypurhat district in Bangladesh by initial screening of AIV type A by ELISA followed by H9 LPAI detection by HI test. 180 sera samples from 18 flocks were collected and screened for Avian Influenza Virus type A antibodies by ELISA. All ELISA positive samples were tested by HI test, specific for H9 antibodies. On ELISA, sero-prevalence for AIV type A were 14.4% and 3.3% in older aged group (≥11 weeks) and growing aged group (≤10 weeks) respectively, with an overall sero-prevalence of 8.89% in Sonali chickens at Joypurhat district. Among, these AIV sero-positive samples, 92.31% of older aged group and 66.67 % of growing aged group chickens found sero-positive to H9 antibodies by HI test with an overall of 87.5%. Out of 180 sera samples, only 14 samples found positive to H9 antibodies, indicating 7.78% sero-prevalence in Sonali chickens of Joypurhat district. This study revealed higher sero-prevalence of AIV antibodies as well as H9 antibodies in older aged group which might be due long time exposure to infections. Sero-prevalence of both AIV type A and H9 LPAI are higher in older aged Sonali Chickens. The findings of the present study suggest that the Overall seroprevalence of AIV type A and H9 LPAI in Sonali Chickens are 8.89% and 7.78% respectively, at Joypurhat district in Bangladesh and also suggest that H9 LPAI virus are in circulation in Sonali chickens at Joypurhat District in Bangladesh.

INTRODUCTION

Commercial poultry farming in Bangladesh is growing rapidly; in the nineties, the total investment in the poultry sector was only Tk 15 billion, but now it is more than Tk 150 billion. This industry has a great potential for boosting the economic growth of the country as well as ensuring food security (Ali and Hossain, 2012**)**.

Avian influenza is an important poultry disease caused by Type A influenza viruses under the family *Orthomyxoviridae*, that can cause mild to severe infection in different avian species resulting in severe damage to the poultry industry. Influenza A viruses infecting poultry are divided into two groups based on their pathogenicity: highly pathogenic avian influenza (HPAI) which cause generalized rather than respiratory disease with flock mortality as high as 100% and low pathogenic avian influenza (LPAI) which usually causes much milder respiratory disease with low mortality if there are no secondary viral and/or bacterial infection or poor environment condition (OIE Manual, 2009).

In 2003 to 2004, HPAI virus caused multiple and widespread poultry outbreaks in many Asian countries, including Cambodia, China, Indonesia, Japan, South Korea, Laos, Malaysia, Thailand and Vietnam. The epizootic subsequently progressed to cause outbreaks in migratory birds and poultry across nearly fifty countries in the rest of Asia, Europe, Middle East and Africa starting in late 2005 and early 2006 (Alexander 2007b). The first outbreak of H5N1 HPAI in Bangladesh was reported in 2007 (Islam *et al*., 2008a; Biswas *et al*., 2008) and since then as many as 549 outbreaks have been reported to OIE as of October 2013 (OIE, 2013) in 492 commercial and 57 backyard poultry farms. So far, there have been seven human cases of H5N1 avian influenza in Bangladesh with one death (WHO, 2013). HPAI is now considered deeply entrenched in poultry of several countries including China, Vietnam, Indonesia, Egypt, Bangladesh and India (FAO, 2011). Stamping-out has been the national policy in Bangladesh in combating H5N1 HPAI. Recently vaccination against H5N1 has been introduced on trial basis.

Non-H5 or non-H7 AI isolates that are not virulent for chickens are identified as LPAI (OIE, 2009). Low pathogenic avian influenza viruses H9N2 became panzootic in the mid-1980s among multiple avian species in Asia, the Middle East, Africa and Europe. The H9N2 viruses infection is generally characterized by mild respiratory infections, however nowadays these viruses produce significant disease problems associated with high mortality in poultry. Although H9N2 viruses are characterized as LPAI viruses, they may cause high morbidity and mortality that may increase the risk of infections of H5N1 HPAI (Park *et al.,* 2001). The zoonotic transmission to human has also been reported (Peiris *et al.*, 1999). Furthermore, the prevalence of H9N2 in poultry provides ample opportunity for acquiring mutations and reassortment (Capua and Alexander 2008; Webster *et al*. 2007). The virus bears ability to take part in reassortment events through donating internal genes to HPAI subtypes (H5, H7) that raised the possibility to generate a new pandemic potential virus strain (Guan *et al.* 1999; Li *et al* 2014). Low pathogenic H9N2 viruses are circulating in poultry farms of Bangladesh (Negovetich *et al*., 2011; Jannat *et al*., 2013; Shanmuganatham *et al*., 2013; Parvin *et al*., 2014). Both HPAIV H5N1 and AIV H9N2 are co-circulating among poultry population in many Eurasian countries mostly reported from Bangladesh, China, India, Pakistan, Vietnam, Israel, Egypt and United Arab Emirates. Such co-circulations might lead to several mutations followed by reassortment which accelerate to generate new AIV strains. The AI outbreaks caused by H5N1 and H9N2 viruses are still ongoing events in Bangladesh and Egypt, especially in commercial and backyard chickens which become geographically widespread across the respective country, causing serious respiratory infections with high mortality rates resulting in severe economic losses. Although the LPAI is circulating in Bangladesh, there are very limited studies on LPAI even not in Sonali Chickens. There are no activities for controlling LPAI in Bangladesh.

The MS research work is aimed to study the Prevalence of Avian influenza type-A as well as LPAI in Sonali Chickens at Joypurhat District.

1.1 Objectives of the MS dissertation work

The specific objectives of the study were as follows:

i) Detection of antibodies to avian influenza type-A in Sonali Chickens in Joypurhat district.
ii) Prevalence study of LPAI in Sonali Chickens in Joypurhat district.

REVIEW OF LITERATURE

2.1 Avian influenza

Avian influenza A viruses belonging to the *Orthomyxoviridae* family, a major cause of acute respiratory diseases and have caused significant disease outbreaks worldwide (Webster *et al.* 2005). Avian influenza (AI), commonly described as `bird flu` resulted in great economic losses (WHO 2014b). Outbreaks of influenza A viruses continue to cause morbidity and mortality worldwide in domestic birds and a wide variety of mammals, including humans. In recent years, a number of AI strains have crossed the species barrier also to humans causing severe clinical signs and even death (Webster *et al.* 2005) and in 1997 a direct avian to human transmission occurred with highly pathogenic avian influenza (HPAI) viruses H5N1 from domestic poultry to humans in Hong Kong (de Jong *et al.*, 1997; Claas *et al.,* 1998). This highly pathogenic avian influenza virus (HPAIV) H5N1 is endemic in Bangladesh, China, Cambodia, Indonesia, Vietnam and Egypt causing significant mortality (CDC 2012). Limited human infections with HPAIV of subtype H7 have been detected (CDC 2013 and LI *et al.* 2014) and less recognized human infections has been associated with the avian influenza virus (AIV) H9N2 (Uyeki *et al.* 2012 and Cheng *et al.* 2011). Although much scientific and public health interest has focused on the HPAI H5N1 viruses, low pathogenic avian influenza H9N2 also demands the scientific research. AIV infect a wild variety of bird species in nature. Wild aquatic birds are generally considered as the primary natural reservoir for AIV worldwide (Webster et al. 1992; Kaleta *et al.* 2005; Fouchier and Munster 2009; Olsen *et al.* 2006). Based on HA (1-16) and NA (1-9) AIV can be categorized into a numbers of subtypes and recently two other subtypes of Influenza A virus, H17N10 and H18N11, are identified in bats (Tong *et al.* 2012; Tong *et al.* 2013). Interspecies transmission of AIV between wild bird populations and domestic poultry species is an occasional event (Suarez 2000). Complex interactions between several viruses and host factors are needed for successful AIV transmission and adaptation to new hosts (Yassine *et al.* 2010). Mutations in the

virus genome as well as reassortment may allow viruses to cross species barriers, adapt to new hosts, and potentially increase virulence (Neumann and Kawaoka 2006).

2.1.1 Historical background

Highly pathogenic AI in chickens was first described in 1878 and Perroncito reported this malady as 'fowl plague' in chickens in Italy (Fenner *et al*., 1975; Horimoto, *et al*., 2001; Swayne and Halvorson, 2003). In 1901, Centanni and Savonuzzi determined the cause as a filterable agent (Swayne and Halvorson, 2003) suggesting its etiology was viral (Alexander *et al*., 2000; Horimoto *et al.,* 2001). Hirst then characterized the hemagglutination activity of influenza virus in 1941, and Schafer further characterized 'fowl plague' as a member of the influenza A virus group in 1955 (Horimoto *et al.,* 2001).

HPAI outbreaks have been known to extend since being first described in Italy. A severe outbreak of HPAI in Northern Italy during 1894 spread to chickens in various parts of Europe (Horimoto *et al*., 2001; Swayne andHalvorson, 2003) where it remained endemic until the mid-1930s (Swayne andHalvorson, 2003). Healthy wild birds, particularly Anseriformes (shorebirds) and Charadniiformes (waterfowl) have been found to be asymptomatic reservoirs of AIV. Avian influenza virus is most commonly introduced into domestic poultry by wild migratory waterfowl (Jacob *et al*., 1998). Fortunately, most AIVs from the wild birds are LPAI viruses (Horimoto *et al*., 2001; Swayne and Halvorson, 2003), and the prevailing understanding is that HPAI emerges only after the virus has been introduced to poultry from wild birds (Alexander *et al*., 2000).

In 1949, the first isolate of low pathogenic avian influenza (LPAI), an H10, was obtained from chickens in Germany (Swayne and Halvorson, 2003). The H9N2 (LPAI) virus was first reported in 1966 in the United States (Homme and Easterday, 1970). Since then, the virus has been isolated and reported many times

from various countries (Alexander, 2003; Senne, 2003) including Hong Kong (in 1975, 1985, 1992, 1994, 1997), China (in 1994), and the Middle East (in 2001). The H9N2 virus was first isolated from chickens and domestic ducks in Jordan in 2003 (Monne *et al.,* 2007). In November 2011, H9N2 infection was 1st reported in Egypt in bobwhite Quail (El-Zoghby *et al.,* 2012). The H9N2 has been recorded in the Middle East region for several years, indicating additional risk factors to the poultry industry.

The first outbreak of H5N1 HPAI in Bangladesh was reported in 2007 (Islam *et al*., 2008a; Biswas *et al*., 2008) and since then as many as 549 outbreaks have been reported to OIE as of October 2013 (OIE, 2013), which occurred in 492 commercial and 57 backyard poultry farms. Bangladesh has confirmed its first case of human infection with H5N1 avian influenza on 28May 2008, and as of 12 March 2013, 7 cases of H5N1 avian influenza virus infection in human in Bangladesh has been reported to WHO including one case fatality (WHO, 2013). AIV H9N2 was isolated from a sample of a poultry farm collected in September 2006 (Parvin et al. 2014a). Low pathogenic H9N2 viruses are circulating in poultry farms of Bangladesh (Negovetich *et al*., 2011; Jannat *et al*., 2013; Shanmuganatham *et al*., 2013; Parvin *et al*., 2014). H9N2 influenza viruses have been isolated from live bird market as well as from poultry flocks in Bangladesh (Negovetich *et al*., 2011; Shanmuganatham *et al*. 2013; Parvin *et al.* 2014a). Human infection with AIV H9N2 was also identified in 2011 (ICDDR'B 2013).

2.1.2 Hosts range

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

known to be especially vulnerable to infection. Other animal species including pigs, ferrets, cats, mink and monkey can be infected experimentally with AI viruses (Hinshaw *et al.,* 1981; Kilbourne 1987). Humans also have been affected with AI viruses (de Jong *et al*., 1997; Claas *et al*., 1998; Subbarao *et al.,* 1998; Yuen *et al*., 1998).

2.1.3 Morbidity and mortality

Morbidity and mortality in chickens and turkeys are variable (Swayne and Halvorson, 2003) and may cause rapid death in chickens and turkeys often approaching 100% within 2 to 12 days after the first signs of illness (Cherbonnel *et al*., 2003; Suarez *et al*., 2004; Trani *et al*., 2006; Alexander 2007a; Capua and Alexander 2009). Whereas with LPAI, there is usually high morbidity, however, mortality can vary widely depending primarily on if secondary infections are present (Swayne Halvorson, 2003). LPAI can cause a localized infection with little or no disease unless complicated by other organism or poor environmental conditions (Suarez *et al.*, 2004,).

2.1.4 Transmission

The wild migratory aquatic birds are suspected to be the prime agents of geographic-scale spread of the pathogen at global level (Kilpatrick *et al*., 2006; Causey and Edwards, 2008) while the infected poultry and ducks are important agents at the landscape level (Kilpatrick *et al*., 2006). Specifically, ducks that migrate annually are likely to spread influenza viruses along the migration routes, primarily by exposing the resident and domestic duck populations at the numerous stopover sites (Arzel *et al*., 2006; Olsen *et al*., 2006; Wallensten *et al*., 2007). AIV H9N2 is wide spread in nature, and is routinely isolated from wild birds and occasionally from pigs and other mammalian species (Yu *et al.* 2011). They generally caused mild illness and become panzootic in the mid-1980s among

chickens, ducks, turkeys, pheasants, quails, ostrich and migratory birds (Knipe and Howley 2007).

The infection cycle among birds depends on faecal-oral transmission chains as well as transmission through aerosols. Apart from being directly transmitted from host to host, indirect spread via virus-contaminated water and fomites is also possible. Transmission is also likely to be occurring between wild and domestic bird populations in both directions (Normile, 2006). Live bird markets (LBM) are common in Asian countries because of a cultural preference to consume freshly slaughtered meat which may also contribute in disease transmission (Webster, 2004; Woo *et al.,* 2006).

2.1.5 Clinical signs in poultry

Influenza A viruses infecting poultry can be divided into two distinct groups based on their ability to cause disease. The very virulent viruses (HPAI) cause mortality which may be as high as 100%. It was believed that the infection with LPAI viruses generally runs a mild or an entirely asymptomatic course as these viruses co-exist in almost perfect balance with the natural hosts (Webster et al. 1992; Alexander, 2000). LPAI viruses cause localized infections, while HPAI is characterized as a systemic disease resulting in high mortality which may reach up to 100% within 48 h in some poultry species (WHO 2014; Swayne and Suarez 2000; Capua *et al.* 2000). Clinical signs of LPAI in chickens include rales, coughing, conjunctivitis and airsacculitis (Ladman et al. 2008) as well as decrease in egg production with the presence of misshapen eggs (Capua *et al.* 2000). LPAIV infections in turkeys are similar to chickens (Alexander *et al.* 1986).

2.1.6 Pathology

Pathological lesions of avian influenza are neither specific nor consistent. Lesions may vary with the viral strain and the species and age of the host. In general, only turkeys and chickens reveal gross and microscopic alterations especially with

strains adapted to these hosts (Capua and Mutinelli, 2001). The critical and important difference between LPAIV and HPAIV is located at the proteolytic cleavage site of HA sequences (Garten *et al.* 1981; Klenk 1980; Lazarowitz *et al.* 1973). HPAIV can be defined as having multiple basic amino acids (PEXPKXR/GLFG), whereas LPAI viruses have mono- or di-basic amino acids (PQRETR/GLFG) at the HA cleavage site (Perdue *et al.* 1997). The HA of HPAI viruses can be cleaved by ubiquitous furin-like or subtilisin-like endoproteases (Garten *et al.* 1981; Horimoto and Kawaoka 1995) which increases the ability of the virus to be cleaved in extra-intestinal and extra-respiratory tissues, leading to systemic viral replication. In contrast, LPAI viruses have restricted tissue tropism because the cleavage of the HA can only be accomplished by trypsin-like proteases limiting replication to the respiratory and intestinal tracts where these enzymes are present (Steinhauer 1999). Gross lesions restricted to the respiratory tract, including tracheitis, bronchitis, airsacculitis and pneumonia (Shalaby et al. 1994; Swayne and Slemons 1994). Histologic signs of LPAI in chickens and turkeys include loss of cilia, heterophilic infiltrate and luminal exudate in the trachea as well as bronchitis and interstitial pneumonia (Ladman *et al.* 2008). Some isolates of LPAIV have been shown to result in renal tubule necrosis, interstitial nephritis, lymphocyte necrosis and depletion in the bursa, spleen and thymus in addition to respiratory pathology (Swayne 1997). Virus can usually be isolated from lung, trachea and air sac epithelium, but also from the oviduct, ovary and tubular epithelium of the kidney with no pathological lesions (Shalaby *et al.* 1994).

2.2 Avian influenza virus

Influenza viruses are members of the *Orthomyxoviridae* family (Lamb and Krug, 1998; Palese and Shaw, 2007) and are enveloped, negative sense, single-stranded ribonucleic acid (RNA) viruses. The virions are pleomorphic: spherical (80-120 nm in diameter) or filamentous forms (Lamb and Krug, 1998; Swayne and Halvorson, 2003; OIE, 2012) and they contain distinct gene segments in helical nucleocapsids, which are surrounded by a matrix protein shell, and an outer lipid envelope derived

from the host cell membrane. Differences in the virus nucleoprotein and matrix protein allow these viruses to be classified under three genera or types, i.e. Influenza virus A, B or C. Influenza A viruses have been isolated from various species, including humans, pigs, horses, mink, seals, whales and birds. Influenza B and C viruses are primarily human viruses (Webster *et al.*, 1992; Swayne and Suarez., 2000; Alexander, 2007a). Influenza A viruses can further be subtyped based on the antigenic relationships of the haemagglutinin (HA) and neuraminidase (NA) surface glycoprotein.To date 18 different types of HA (H1–H18) and 11 NA(N1–N11) have been recognized (Fouchier *et al*., 2005; CDC, 2007; Swayne , 2008; OIE, 2012; Tong *et al*., 2012; 2013). The newly described H17N10 and H18N11 subtypes are the influenza viruses of bats (Tong *et al*., 2012; 2013).

2.2.1 Taxonomy

The current taxonomic position of Influenza A viruses is as follows:

Family: *Orthomyxoviridae*

Genus: *Influenzavirus A*

Species: *Influenza A virus*

The World Health Organization (WHO) guidelines (Anonymous, 1980) for the nomenclature of influenza viruses are as follows. First, the type of virus is designated (A, B or C), then if non-human, the host name, followed by the place of isolation, the isolate number and the year of isolation each separated by slashes. For influenza A viruses, the hemagglutinin (HA) and neuraminidase (NA) subtypes are noted in parenthesis.

2.2.2 Virus structure and genome

The structure of influenza virus is presented schematically in Fig. 1. Avian influenza viral genome is composed of eight segments of single-stranded, negative sense (complementary to mRNA) RNA molecules (Easterday *et al.*, 1997;

Horimoto and Kawaoka, 2001). Those eight segments (HA, NA, NP, M1, M2, PB1, PB2 and PA) code for 10 viral proteins, eight of which are constituents of the virions (Easterday *et al.,* 1997), whereas a ninth one NS2 is also now known to exist in virions in association with M1 (Richardson and Akkina, 1991; Yasuda *et al.,* 1993; Horimoto and Kawaoka, 2001). NS1 is the only non-structural protein of influenza A viruses. Size and functions of these proteins are summarized in table-1.

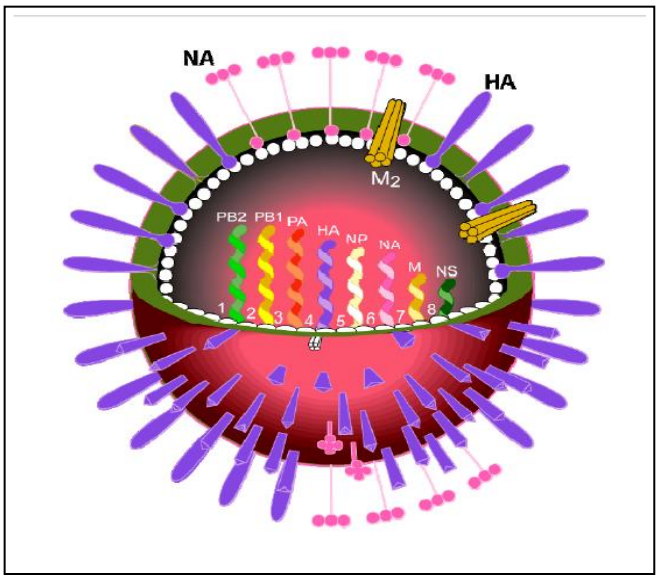

Fig. 1**:** Schematic diagram showing the structure of Influenza Virus

HA: Hemagglutinin, NA: Neuraminidase, M1: Matrix proteins 1, M2: Matrix proteins 2, PB1: Basic polymerase 1, PB2: Basic polymerase 2, PA: Acidic polymerase protein, NP: nucleoprotein, NS: Non-structural protein (Webster, 2002).

Table – 1: Genome segment, coding proteins and their main functions

Segment	Protein name	Encoded polypeptide	Length (nt)	Functions
PB2	PB2	Polymerase basic 2	2341	Component of RNA polymerase, cap recognition
PB1	Polymerase basic 1	PB1, PB1-F2	2341	Component of RNA polymerase, endonuclease activity, elongation
PA	Polymerase acidic	PA	2233	Component of RNA polymerase, protease
HA	Hemagglutinin	HA	1778	Surface glycoprotein, receptor binding, fusion activity, major antigen
NP	Nucleoprotein	NP	1565	RNA binding, RNA synthesis, RNA nuclear import
NA	Neuraminidase	NA	1413	Surface glycoprotein, neuraminidase activity
M	Matrix	M1, M2	1027	M1: interaction with vRNPs and surface glycoprotein, nuclear export, budding M2: ion channel activity, assembly
NS	Nonstructural	NS1, NS2 (NEP)	890	NS1: multi-functional, viral IFN antagonist NS2/NEP: nuclear export of vRNPs

2.2.2.1 Polymerases (PB2, PB1 and PA)

PB1, PB2 and PA are the three subunits of viral polymerase that participate in RNA replication and transcription in conjunction with NP (Easterday *et al.*, 1997; Horimoto and Kawaoka, 2001). These polymerase proteins are encoded by the three largest viral RNA segments. Collectively, these three polymerase proteins form a complex in the cytoplasm and nucleus of the host cell (Krieg *et al.*, 1998; Lamb and Krug, 1998). These proteins are synthesized in the cytoplasm and due to their intrinsic karyophilic properties, are transported to the nucleus; each of the proteins contains a nuclear localization signal (Lamb and Krug, 1998). The PB1 protein is concerned in the recognition and snatching of the 7-methylguanosine cap

of the 5' end of host cell mRNA (Krieg *et al*., 1998). It is also a part of the transcriptase complex (Krieg *et al.,* 1998; Lamb and Krug 1998). PB2 has endonuclease activity and catalyzes nucleotide addition (Lamb and Krug 1998; Swayne and Halvorson, 2003). It is also a part of the transcriptase and replication complex (Lamb and Krug 1998; Swayne and Halvorson, 2003). The PA protein is involved in viral genomic RNA replication and has proteolytic activity (Flint *et al.,* 2000; Lamb and Krug 1998; Swayne and Halvorson, 2003).

2.2.2.2 Hemagglutinin

Haemagglutinin (HA) enables the virion to attach to host cell surface sialyloligosaccharides (Paulson, 1985) and is accountable for its hemagglutinating activity (Hirst, 1941). HA is responsible for eliciting the production of virus neutralizing antibodies that are important in protection against infection (Easterday *et al.,* 1997).HA is the means by which the virus first initiates its propagation in the host, it also serves as a major target by which the host keeps this virus in check from being further transmitted. It was so named because of its ability to agglutinate erythrocytes (Lamb and Krug 1998). The HA has three major roles (Lamb and Krug 1998): provides virus attachment to host receptors (Humiston andPappano2004; Skehel and Wiley., 2000; Swayne and Halvorson, 2003), mediates fusion between the virus and endosomal membranes that results in the release of viral RNA into the cytoplasm (Lamb and Krug 1998) and act as the major antigenic protein against which neutralizing antibodies are produced (Lamb and Krug 1998; Swayne and Halvorson, 2003). It is the protein against which the vaccines are targeted.

The HA gene on RNA segment four was the first influenza gene to be fully sequenced and it encodes for 18 different known subtypes (Lamb and Krug 1998; Fouchier *et al.,* 2005; Tong *et al*., 2012; 2013). The 16th subtype was described in 2005 (Fouchier *et al.,* 2005) and the 17th and 18th subtype in 2012 and 2013 (Tong *et al.,* 2012; 2013).

HA is synthesized on membrane-bound ribosomes and exists as the HA0 precursor protein made up of HA1 and HA2 subunits (47 kDa and 29 kDa, respectively) linked together by disulfide chains (Lamb and Krug 1998; Skehel *and* Wiley., 2000). The trimeric structure is divided into two regions: the stalk and the globular head (Lamb and Krug 1998). The stalk contains the HA2 part and is proximal to the membrane and the membrane-distal head contains the HA1 part (Lamb and Krug 1998).

2.2.2.3 Nucleoprotein

Nucleoprotein (NP) encoded by segment – 5, is contained within the viral envelope in association with the eight RNA segments of the virus and is a part of the ribonucleoprotein (RNP) complex that participates in RNA replication and transcription (Horimoto and Kawaoka, 2001). The NP is the primary structural protein that interacts with each viral RNA segment to form the RNPs (Lamb and Krug 1998). It is a type-specific antigen in that the different types (A, B or C) of influenza viruses, which can be distinguished based on this protein (Lamb and Krug 1998). NP molecules carry out various functions in infected cells (Swayne and Halvorson, 2003). They are involved in transporting vRNPs from the cytoplasm to the nucleus of an infected cell and are necessary for the synthesis of full-length vRNA (Lamb and Krug 1998). NP molecules are one of the major targets for cytotoxic T lymphocytes (Lamb and Krug 1998; Ulmer *et al*., 1998). DNA vaccine studies involving NP DNA have shown to induce both CD4+ and CD8+ T cells and elicit limited heterosubtypic protection against influenza challenge (Ulmer *et al*., 1998).

2.2.2.4 Neuraminidase

The neuraminidase (NA) is another vital membrane protein found on the influenza virus particle that is used for subtyping of the virus (Lamb and Krug 1998; OIE 2012; Palese and Shaw, 2007). NA is a sialidase (Gottschalk, 1957) and it prevents virion aggregation by removing cell and virion surface sialic acid (Paulson 1985).

Antibodies to NA are also important in protecting hosts (Webster *et al.*, 1992). NA has two important functions: (1) it remove sialic acid from glycoproteins (*i.e.* HA, NA and cell surfaces), prevent influenza viruses from attaching to each other or remaining bound to cells, which would effectively decrease the level of infectious viral particles; (2) it is an antigenic determinant, which undergo antigenic deviation (Lamb and Krug 1998). These proteins may also allow the virus to move through the respiratory tract's mucin layer and find the target epithelial cells (Harvey *et al*., 2004; Lamb and Krug 1998). Eleven protein subtypes have been identified for NA (Lamb and Krug 1998; Tong *et al., 2*012).

2.2.2.5 Matrix protein

The matrix (M1) protein is the most abundant protein in the virus particle and lies just beneath the virus envelope, providing structural support (Lamb and Krug 1998). The ion channel (M2) protein is not as abundant in the virion but it serves an important function; it allows the influx of hydrogen ions (H+) into the virus particle while the virus is in the endosome (Lamb and Krug 1998). This ultimately results in a drop in pH inside the virion that results in a conformational change in the HA (Lamb and Krug 1998).

2.2.2.6 Non-structural protein

The smallest segment of viral RNA, segment eight, encodes for two nonstructural proteins, NS1 and the nuclear export protein (NEP) and NS2 (Lamb and Krug 1998; Palese and Shaw, 2007). The NS2 mRNA was the first proof that splicing occurs with an RNA virus that lacks any DNA intermediates during replication (Lamb and Krug 1998). The NS1 protein exists abundantly in the nuclei of infected cells (Lamb and Krug 1998; Tumpey *et al.,* 2005), where they interact with polysomes. This protein regulates many host cell functions such as suppression of innate immunity by preventing host cell mRNA processing (Nemeroff *et al.*, 1998) and preventing export of polyadenylated cellular transcripts from the nucleus (Qiu *et al.*, 1994; Satterly *et al.,* 2007). The NS1 protein is also important in endowing

the virus with resistance to host cell interferon (Lamb and Krug 1998; Garcia-Sastre, 2001; Haller *et al*., 2006).

2.2.3 Pathotypes and sub-types

Influenza viruses are divided into three types: Type A, Type B and Type C or three genera *Influenzavirus A, Influenzavirus B* and *Influenzavirus C*. The types or genera are distinguished by the antigenic differences in the nucleoprotein (NP) and matrix (M) proteins. Influenza A viruses infects humans; several other mammalian species and a wide range of avian species (Webster *et al.*, 1992). Influenza B viruses infect humans and have been isolated from seals (Osterhaus *et al.*, 2000). Influenza C viruses have been mainly isolated from humans but isolation from pigs has been reported (Guo and Desselberger, 1984). Based on two surface glycoprotein: hemagglutinin (HA) and neuraminidase (NA), Type A influenza viruses can be divided into subtypes. Currently, 18 HA and 11 NA subtypes are known (Fouchier *et al*., 2005; CDC, 2007; Swayne 2008; OIE, 2012; Tong *et al*., 2012; 2013).

Avian influenza viruses differ in their virulence and have been grouped into two pathotypes: low pathogenic avian influenza (LPAI) and highly pathogenic avian influenza (HPAI) viruses.

2.2.4 Evolution of avian influenza virus

Avian influenza virus constantly adapts to its hosts through evolution of its gene constellation. The lack of proof-reading of its RNA polymerases results in error prone replication and high rates of point mutations (antigenic drift) allowing the influenza virus to evade host immune responses (Webster and Hulse, 2004).

Influenza viruses are perpetuated and have remained largely benign in aquatic birds such as ducks, gulls and shorebirds. These birds are regarded as the primordial source of influenza viruses for other avian and mammalian species (Webster *et al.*, 1992; Swayne and Suarez, 2000; Alexander, 2007a). Virulent viruses normally

arise through passages of non-pathogenic strains in domestic poultry, as these viruses are not typically maintained as highly pathogenic forms in wild birds.

2.3 Diagnosis of avian influenza

Disease diagnosis is the prerequisite for controlling and eradicating a disease. Diagnosis of avian influenza depends on history, clinical observation, gross and microscopic pathology, direct demonstration of virus or antigen, and indirect measurement of viral exposure through antibody detection (Swayne and Suarez, 2000). A combination of virus isolation, serological tests, and direct antigen detection is often used to detect infected flocks .WHO and Office International des Epizooties (World Organisation for Animal Health; OIE) recommended diagnostic techniques for AIV include enzyme-linked immunosorbent assay (ELISA), haemagglutination inhibition (HI), neuraminidase inhibition (NI) tests, egg and tissue culture inoculation, agar gel immunodiffusion (AGID) and both real-time and conventional reverse transcriptase PCR (RT-PCR) as newer molecular methods (OIE, 2012; WHO, 2012). As a gold standard, IAVs have been categorized using the ELISA with HA and NA subtypes that depends on specific antibodies. Subsequent to the initial confirmation of an index case, virus isolation in outbreak investigations can be replaced by nucleic acid detection tests - conventional or real-time reverse transcription-polymerase chain reaction (RT-PCR), or nucleic acid sequence-based amplification (NASBA) assays (Collins *et al.*,2002; Suarez *et al.*,2007).

2.3.1 Virus Isolation

Conventionally, AI virus is isolated by inoculation of swab fluids or tissue homogenates into 9- to 11-day-old embryonated chicken eggs, usually by the allantoic sac route. For HPAI viruses, virus isolation procedures are much quicker that kills embryos in 30 to 72 hours and can be subtyped by HI tests within 2-3 days. (WHO, 2012). Depending on the pathotype, the embryos may or may not die

within a five-day observation period and usually there are no characteristic lesions to be seen in either the embryo or the allantoic membrane (Mutinelli, 2003b). Haemagglutination (HA) is an insensitive technique requiring at least $10^{6.0}$ particles per ml. If only a low virus concentration is present in the inoculum, up to two further passages in embryonated eggs may be necessary for some LPAI virus strains, in order to produce enough viruses to be detected by HA (Kamps *et al*., 2006).

2.3.2 Agar Gel Immunodiffusion Test

Avian influenza virus can be identified as influenza A virus with agar gel immunodiffusion (AGID) test. AGID tests can recognize all avian influenza subtypes in poultry, but the test is relatively less sensitive. AGID tests are not reliable for detecting avian influenza in ducks or geese (Swayne, 2008).

2.3.3 Enzyme immunoassay and immunochromatography

Enzyme immunoassays (EIAs) or immunochromatography assays utilize antibodies directed against viral antigen that are conjugated to an enzyme. An incubation step with a chromogenic substrate follows and a color change is indicative of the presence of viral antigen. Certain enzyme immunoassays as well as similar assays using immunochromatography allow for bedside/penside testing (Allwinn *et al.*, 2002) taking 10-30 minutes. Sensitivities of EIAs vary between 64% and 78% (Allwinn *et al*., 2002). Different rapid tests can detect either influenza A or B virus without distinguishing the type or influenza A virus only, or detect both influenza A and B and identify the type. However, none of these rapid tests can differentiate between subtypes that infect humans (H1N1 and H3N2) or avian influenza subtypes (FDA, 2012). World Organization for Animal Health (OIE) recommended that antigen detection tests be used to identify avian influenza only in flocks and not in individual birds (Suarez *et al*., 2007; OIE, 2012).

Competitive ELISA systems (cELISA) have been implemented to detect antibodies against AI NP in different avian species (Shafer *et al*., 1998; Starick *et al*., 2006). This refined species independent approach has been used for wildlife surveillance (de Marco *et al.,* 2003).

2.3.4 Immunohistochemistry

Immunohistochemistry (IHC) has been used to detect avian influenza virus antigen in tissue sections (Rimmelzwaan *et al.,* 2001; Gu *et al.*, 2007; Kalthoff *et al*, 2008; Chen *et al.*, 2009) in cojunction with hematoxylin and eosin staining on histopathology sections (Kuiken *et al.,* 2003; Perkins and Swayne, 2003; Klopfleisch *et al.*, 2006; 2007; Teifke *et al.*, 2007; Vascellari *et al.*, 2007). Immunohistochemical methods are commonly used for studying the pathogenesis of the avian influenza virus by allowing the identification of sites of replication of the virus in infected tissues and the correlation with the histopathological changes observed. Viral antigens in fresh, frozen, or fixed cell suspensions or tissues can be detected by the use of specific antibodies labeled with dyes or enzymes. Antigen detection can then be related to the lesions observed and the stages of the infection (Ellis *et al*., 2002; Perkins and Swayne, 2001).

2.3.5 Reverse transcription polymerase chain reaction (RT-PCR)

A more rapid approach, especially when exclusion of infection is demanded, employs molecular techniques, which should also follow a cascade style: the presence of influenza A specific RNA is detected through the RT-PCR which targets fragments of the M gene, the most highly conserved genome segment of influenza viruses (Fouchier *et al*., 2000; Spackman *et al*., 2002), or the nucleocapsid gene (Dybkaer *et al.*, 2004). If a positive result is obtained, RT-PCRs amplifying fragments of the subtype-specific hemagglutinin gene are run (Spackman *et al*., 2002; Dybkaer *et al.,* 2004) and then pathotyping is done by

sequence analysis a fragment of the HA gene spanning the endoproteolytic cleavage site.

2.4 Serological surveillance of Avian Influenza in poultry

Surveillance is an essential foundation for monitoring and evaluating any disease process and is especially critical when new disease agents appear. H9N2 viruses are endemic in terrestrial poultry (eg, chicken, qual) and are geographically widespread throughout Asia (Cameron et al., 2000; Guan et al., 2000), LPAIV H9N2 subtype has been isolated worldwide from different types of terrestrial poultry (Cameron *et al.*, 2000; Li *et al*., 2003). Initially concentrated in Asia (Capua and Alexender, 2004), outbreaks subsequently spread to Africa, the Middle East and America (Homme and Easterday, 1970) causing significant economic losses related to increased mortality and decreased production in poultry industry (Swayne and Halvorson, 2003). It has also been reported that H9N2 avian influenza virus can cross species barrier and infect humans (Peris *et al.*, 1999) although only causing mild flu-like respiratory illness (Butt *et al*., 2005; Guo et al., 2000; Lin *et al*., 2000; Peris *et al*., 1999). Monitoring AI viral infections in domestic and wild birds is therefore important to control animal diseases and prevent human pandemics. Many state laboratories participate in the surveillance of AI activity and contribute to the early recognition of newly emerging epidemic strains (Naeem *et al*., 2003). Serological surveillance of antibodies against AIV is of great importance in preventing and controlling AI infection. Identification of both H and N subtypes is highly essential for epidemiology. Nowadays, the majority of field surveys of LPAIV are based on serological assays; molecular methods such as real time reverse transcription PCR (rRT-PCR), which have been proven superior regarding its sensitivity and suitability for high throughput analyses.

Virus isolation and identification, serological tests, such as agar gelimmunodiffusion, enzyme-linked immunosorbent assay (ELISA), and hemagglutination inhibition (HI) test, have been widely used for AI surveillance

((Swayne & King, 2003; Lee & Saif, 2009). Scientist have employed ELISA in different format for sero-surveilance of Type -A avian influenza and HI for LPAI H9 (Hadipour, 2010; Pawar *et al.*, 2012). The haemagglutination inhibition (HI) test is an accurate, inexpensive and potent test, which render it the most commonly used tests to determine the presence of antibodies in the serum to an influenza virus.

Seroprevalence of AIV might be corelated with the rearing system, scope for entrance to common water reservoir, direct or indirect contact with migratory route (Alexander, 2003; De Marco *et al*., 2003; Senne *et al*., 2003; Vander *et al*., 2003; Capua & Alexander, 2004). Cheng *et al.* (2002) found H9N2 avian influenza antibody titers in 26% of human sera and only in 7% of chicken sera, and concluded that human H9N2 virus infection would probably derived from chicken H9N2 virus. Li *et al*. (2004) reported anti H9N2 antibodies in 12.8% of the chickens and 5.1% of the poultry-farm workers. In Hong Kong during 2001-2003, Choi et al. (2004) reported highest prevalence of H9N2 avian influenza among live poultry markets. Al-Natour *et al*. (2005) reported AIV seroprevalence of 71% among broiler-breeder flocks in Jordan. In Bangladesh, Nooruddin *et al.* (2006) reported an overall 9.82% seroprevalence of avian influenza in native chickens. In Iran, Hadipour (2010) recorded 72.98% seroprevalence prevalence in village chickens in his study areas and Pawar *et al.* (2012) reported 4.7% seropositive poultry workers by HI test in his study areas.

MATERIALS AND METHODS

3.1 RESEARCH AREA

Samples were collected from Sonali chicken raring farms at Joypurhat and most of the laboratory research work was conducted in the Central Disease investigation Laboratory (CDIL), Department of Livestock services, Dhaka.

Figure 2: Working Areas are show in the map by special circle

3.2 RESEARCH PERIOD

The duration of experiment was 6 months from January to June, 2015.

3.3 MATERIALS

Materials used in the present research work, including the equipment, samples and reagents are documented in this chapter. Experimental designs and detailed methodologies are also described. The details of the material and methods are described below:

3.3.1 Equipment

Balance (AND, USA); Microcentrifuge (Eppendorf, Germany) Bench centrifuge; ClassII Biosafety cabinet (ESCO, Singapore); Micropipettes (Eppendorf, Germany); pH meter; Vortexer; Benchtop autoclave; Refrigerators, Freezers, ELISA Reader (Multiskan-EX Lab system, Thermo-scintific) etc.

Figure 3: Microplate Strip Washer (Left) and Microplate Spectrophotometer (Right)

3.3.2 Plastic ware and other consumables

Pipette tips (Eppendorf, Germany); falcon tubes: 15 ml (Becton Dickinson Labware, USA and Eppendorf, Germany); sterile filter tips (Eppendorf, Germany); syringe and needle (Opsonin, Bangladesh), etc.

3.3.3 Sera samples

A total of 180 blood samples of Sonali chickens of different age groups were collected from farms of Joypurhat District in this study and subjected to ELISA and HI test.

3.3.4 Test kits and reagents

The test kits and antigen used in this study are listed in Table-.

Table-2: Test kits and Antigen used in the present study

Test kit	Use	Source
Avian Influenza Antibody test ELISA kit	Type-A AIV antibody detection based on Matrix protein	IDEXX(U S A)
H9 antigen	HI test to detect H9N2 antibody in serum	Newcastle and Avian influenza Reference Laboratory, Italy

Apart from these test kits, the following additional reagents were also used:

Figure 4: ELISA Plate (Covered)

Figure 5: Chemicals and Solutions supplied with ELISA Kit

3.3.4 Solutions, buffers and chemicals

1X PBS, Alsever's solution, 1% Chicken RBC were used in the present study.

Phosphate buffered saline (PBS)

A 10X concentration of Dulbecco's phosphate buffered saline solution A (PBS-A) was prepared as follows:

Sodium chloride (NaCl)	*80.0g*
Potassium chloride (KCl)	*2.0g*
Disodium hydrogen phosphate-anhydrous (Na_2HPO_4)	*11.5g*
Potassium dihydrogen phosphate (KH_2PO_4)	*2.0g*
Double distilled water (to make)	*1000ml*

All ingredients were dissolved in distilled water and pH was adjusted to 7.2-7.4 with a pH-meter by adding 0.1M NaOH or 0.1N HCl. The prepared PBS was distributed in screw-capped media bottle in 50ml aliquots and sterilized by autoclaving for 15 min at 121^0c under 15lbs pressure per square inch. Before use, 50 ml PBS (10X) was mixed with 450ml sterile double distilled water to prepare 1X PBS.

Alsever's solution

A 1X concentration of Alsever's solution was prepared as follows:

Sodium chloride (NaCl)	*4.2g*
Sodium Citrate.2H_2O	*8.0g*
Citric Acid.H_2O	*0.55g*
D Glucose	*20.5g*
Double distilled water (to make)	*1000ml*

All ingredients were dissolved in distilled water and pH was adjusted to 6.8 to 7.0 with a pH-meter by adding 0.1M NaOH or 0.1M Citric Acid. The prepared Alsever's solution was prepared in screw-capped media bottle and sterilized by autoclaving for 15 min at 121^0c under 15 lbs pressure per square inch.

Preparation of 1% Chicken RBC

1% chicken RBC was prepared as follows;

- 3ml chicken blood was collected in a falcon tube containing equal volume of alsever's solution.
- Collected blood was gently mixed and centrifuged at 3000 rpm for 10 minutes.
- Supernatent was aspirated and washed with PBS for 3 times.
- Finally, sterile PBS was added to prepare 1% (v/v) chicken RBC and stored at 4^0C.

3.4 METHODS

3.4.1 Blood collection and Sera separation

- 1ml of each of 180 blood samples from Sonali chickens were collected in 3ml disposable syringe and lebelled with separate numbering. Samples were collected from those Sonali chicken rearing farms which have previous history of respiratory illness at Joypurhat.
- The syringe containing blood samples were then kept in horizontal position

at room temparature overnight to separate serum from the collected blood.

- Sera samples were then transferred to separate eppendorf tube (1.5 ml) and subjected to low speed centrifuge at 1000 rpm for 5 minutes and supernatant were collected as serum samples in separate labeled eppendorf tube.
- Separeted serum samples were then stored at -20^{0}C until use.

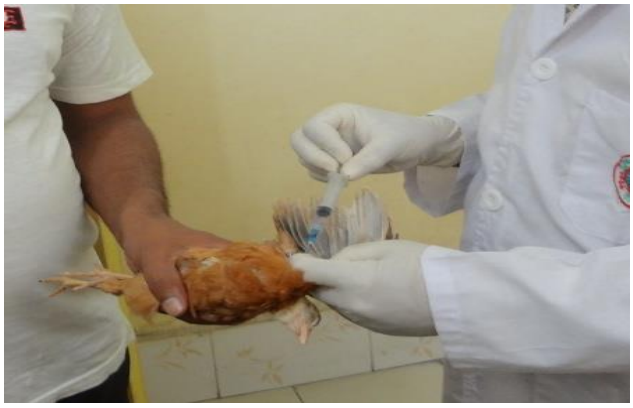

Figure 6: Collection of blood from sonali chicken

Figure 7: Sera sample collected from chicken blood

3.4.2 ELISA test for AIV type – A antibody

The ELISA Kit was obtained from Biocheck Compani. ELISA test was done following manufacturer's instruction. Briefly,

- 100µl of undiluted negative and positive control were added into well A1, B1 and C1, D1 respectively.
- Then 100 µl of diluted collected serum samples (1:500) were added into appropriate wells and recorded in notebook properly.
- Then ELISA plate was covered with lid and incubated at room temperature for 30 minutes.
- After 30 minutes incubation, the contents of the plate were aspirated and washed 4 times with wash buffer (300 µl for each well for each washing).
- Then the plate was tapped firmly on absorbent paper to remove liquid, if any.
- Then 100µl of conjugate reagent were added into each well, tilted, covered with lid and incubated for 30 minutes at room temparature.
- The plate was then again washed 4 times with wash buffer, tapped on absorbent paper and liquid, if any, were removed.
- 100µl of substrate was then added into each of the well, tilted, covered with lid and incubated at 37^0C for 15 minutes.
- Then 100µl of stop solution was added into each of the well and a colour change to yellow was observed.
- The plate was then (as early as possible after adding stop solution) placed in a ELISA plate reader and absorbance value/reading were taken at 405nm and recorded.

3.4.3 Calculation of Result:

The relative levels of antibodies in the unknown samples were determined by calculating sample-positive (S/P) ratio. The equation of calculation provided in ELISA kit was used for the calculation of antibody titer.

a) Negative control mean (NC$\bar{X}$)=

$$\frac{wellA1 + wellA2}{2}$$

b) Positive Control mean (PC$\bar{X}$)=

$$\frac{wellB1 + wellB2}{2}$$

c) S/P ratio=

$$\frac{\text{Sample mean} - NC\,\bar{X}}{PC\bar{X} - NC\bar{X}}$$

d) Titre=

Relates S/p at a 1.500 dilution to an end point titre

$^{Log}{}_{10}$ titre = 1.1($\log_{10}$ S/P) + 3.156

Interpretation of result:

A serum samples with S/P ratios of less than or equal to 0.5 considered negative. S/P ratios greater than 0.5 (titer greater than 668) considered as positive and indicate exposure to AIV.

3.4.4 Hemagglutination (HA) test for preparing of 4HAU antigen

- 25 µL of PBS was added to each of the well in 96 well V-bottomed plastic microtiter plate.
- Then 25µl of antigen (H9N2) was added at first row and serial two fold dilution was done upto 11th well and 12th well was kept as control.
- Again, 25 µL of PBS was added to each of the well in 96 well V-bottomed plastic microtiter plate.
- 25 µL freshly prepared 1% (V/V) Chicken RBC was then added into each well and mixed by tilting and kept at room temperature for 30 minutes.

- The highest dilution of antigens that completely agglutinate the chicken RBC was recorded as 1 HAU.
- 4HAU was prepared with PBS for the use on Hemagglutination Inhibition test.

3.4.5 Hemagglutination Inhibition (HI) test for H9 Subtype detection

HI test was done as per instruction outlined in OIE manual. Briefly,

- 25 µL of PBS was added to each of the well in 96 well V-bottomed plastic microtitre plates.
- Then 25µl of ELISA positive sera was added into the first well of each row separately and recorded properly.
- Two-fold serial dilutions of sera were made across the row up to 11^{th} well and 12^{th} well kept as control.
- 25 µL of 4HAU avian influenza virus subtybe H9N2 was added into each well up to 11^{th} well in each row and incubated at room temperature for 30 minutes.
- 25 µL freshly prepared 1% (V/V) Chicken RBC was then added into each well and mixed by tilting and kept at room temperature for 40 minutes.
- The highest dilution of the test sera that completely inhibited the RBC agglutination was recorded as antibody titre against H9N2.
- Titer of H9 antibody in the test sera $\geq \log_2 4$ was considered as positive (Ghaniei *et al.*, 2013).
- The titer was analysed statistically by measuring arithmetic mean titer (AMT).

RESULTS

4.1 Sero-prevallence of Avian Influenza type A by ELISA

Sero-prevalence of avian influenza type A in sonali chickens of Joypurhat district are shown below in table-3

Age group	No of samples tested	No of ELISA positive samples	No of ELISA negative samples	Sero-prevalence (%)	Over all sero-prevalence (%)
≤10 weeks	90	3	87	3.3	8.89
≥11 weeks	90	13	77	14.4	
Total	180	16	164		

The present study revealed that 3 out of 90 samples of ≤10 weeks aged group were positive and 13 out of 90 samples of aged group ≥11 weeks were positive. The sero-prevalance of avian influenza type A was 3.3% and 14.4% in ≤10 weeks aged group and ≥11 weeks aged group respectively. The over all sero- prevalence of AIV antibodies in sonali chicken at Joypurhat district was 8.89%.

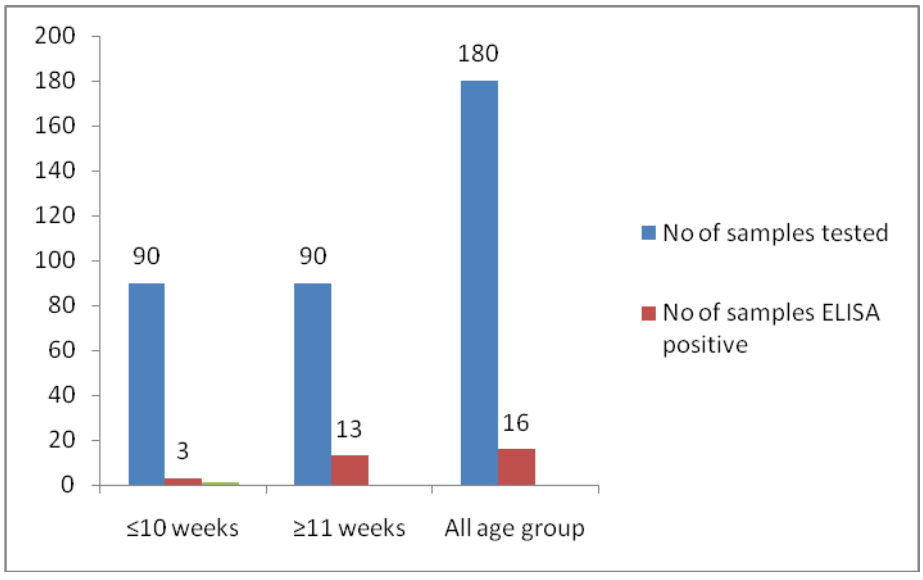

Figure 8: Sero prevalence of AIV type A in different age group of Sonali Chickens at Joypurhat district

Light Yellow Colour Shows Positive of ELISA

Figure 9: ELISA Plate

4.2 Hemaglutination Inhibition test:

All 16 AIV type A positive sera on ELISA test were subjected to HI test. 14 samples out of 16 samples were positive on HI test with HI titer > $\log_2^4$. Results are presented in Table-4.

Age group	ELISA positive Sample	HI titer (Log_2)	AMT	%CV
≤10 weeks	1	3	4.33	35.2
	2	4		
	3	6		
≥11 weeks	1	6	5.62	26.7
	2	4		
	3	8		
	4	5		
	5	6		
	6	7		
	7	4		
	8	7		
	9	6		
	10	7		
	11	6		
	12	4		
	13	3		

DISCUSSION AND CONCLUSION

AIV causes frequent epidemics and occasional pandemics in various animals and thus present a significant public health problem associated with considerable economic consequences. AIVs of various subtypes are circulating in poultry (Abbas *et al.* 2010; Jeong *et al.* 2010; Kim *et al*. 2010). In particular, H5N1 and H9N2 AIVs are predominant among poultry flocks causing severe disease outbreaks with high morbidity and mortality (Nagarajan *et al.* 2009; Xu *et al*. 2007; Cameron *et al*. 2000) in many countries including Bangladesh.

H9N2 has been reported from different countries including Iran (Norouzian *et.al*. 2012), and this subtype is enzootic throughout Asia (Capua and Alexander, 2009). H9N2 viruses are not highly pathogenic for poultry, although opportunistic pathogens and immunosuppressant.

Serological tests are useful for early detection and surveillance of infection. In this regard, four major tests agar gel immunodiffusion, ELISA, HI, neuraminidase inhibition can be used (Swayne *et.al*.2008). HI is more specific and more commonly used diagnostic laboratory test for detection of infection.

Thus, the present study was designed to know the sero-prevalence status of H9 antibodies in Sonali chickens at Joypurhat district in Bangladesh by initial screening of AIV type A by ELISA followed by H9 LPAI detection by HI test.

180 sera samples from 18 flocks were collected and screened for AIV type A antibodies by ELISA. ELISA positive samples were subjected to HI test, specific for H9 antibodies. On ELISA, sero-prevalence for AIV type A were 3.3% and 14.4% in growing aged group (≤10 weeks) and older aged group (≥11 weeks) respectively, with an overall sero-prevalence of 8.89% in Sonali chickens at Joypurhat district. Among, these AIV sero-positive samples, 66.67 % of growing aged group and 92.31% of older aged group chickens found sero-positive to H9 antibodies by HI test with an overall of 87.5%. Out of 180 sera samples, only 14

samples found positive to H9 antibodies, indicating 7.78% sero-prevalence in sonali chickens of Joypurhat district. This study revealed that sero-prevalence of AIV antibodies as well as H9 antibodies is higher in older aged group which might be due long time exposure to infections as the virus is in circulation since 2006 (Parvin *et al*. 2013). Similar studies was done by Nooruddin *et al*. (2006) on native chicken of Bangladesh and found over all 8.92% sero-prevalence of AIV antibodies. In another studies, conducted by Alam *et al.* (2003) found 14% sero-positive native birds in Bogra district. Findings of both studies are nearly close to the present study, although, Alam *et al*. (2010) recorder higher sero-prevalence in other dictrict of Bangladesh (Cox's Bazar 38.60% and Barishal 32.30%).Cheng *et al.* (2002) found 7% H9 sero-positive chicken and Li *et al*. (2004) found 12.8% H9 sero-positive chickens in their study areas. Antibody against Avian influenza may be found at any age (OIE, 2013) of birds. So, the present study may be concluded as;

a) Sero-prevalence of both AIV type A and H9 LPAI are higher in older aged Sonali Chickens.
b) H9 LPAI are in circulation in Sonali chickens at Joypurhat District in Bangladesh.
c) The overall seroprevalence of AIV type A and H9 LPAI in Sonali Chickens are 8.89% and 7.78% respectively, at Joypurhat district in Bangladesh.
d) Further studies can be taken to find out the LPAI scenario in Bangladesh.

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