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ORIGINAL ARTICLE

Potential risk factors of avian influenza virus infection in asymptomatic commercial chicken flocks in selected areas of Bangladesh during 2019

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ABSTRACT

Objective: Avian influenza is a zoonotic disease with a pandemic potential that can infect avian and mammalian species, including humans. Studies aimed at investigating avian influenza virus (AIV) status in asymptomatic chickens and their shedding are uncommon in Bangladesh. Therefore, the current study aimed to examine the distribution of AIV subtypes in asymptomatic commercial chicken flocks and to identify the possible risk factors associated with this infection in two selected sub-districts of Bangladesh.

Materials and Methods: A total of 582 oropharyngeal swabs were collected from 23 chicken farms during 2019 and evaluated for the presence of AIV and its subtypes by real-time reverse transcription PCR assays. Risk factors associated with AIV infection were analyzed from questionnaire data.

Results: Overall, AIV prevalence was 7.73% (n = 45) with 7.39% and 7.92% in Dhamrai and Gazipur Sadar sub-districts, respectively. In AIV-positive samples, the prevalence of A/H5N1, A/H5N2, A/H9N1, and A/H9N2 was 31.11%, 28.89%, 6.67%, and 8.89%, respectively. None of the samples were positive for N6 and N8. The odds ratio (OR) of AIV infection was 1.15 in broiler versus layer and 2 in *Sonali* versus layer chickens. The OR was 1.95 for medium versus small, 2.6 for large versus small flock size, 1.5 for moderate versus good biosecurity, and 2.92 for poor versus good biosecurity practicing farms.

Conclusion: The results demonstrated that A/H5N1, A/H5N2, A/H9N1, and A/H9N2 are circulating in asymptomatic chickens of selected areas. Strict farm biosecurity practices and avoiding higher flock density are recommended to prevent AIV spread in the study.

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Introduction

Avian influenza virus (AIV) is a type A influenza virus belonging to the Orthomyxoviridae family, which is highly fatal, economically important, and with public health implications [1]. The significant clinical signs of AIV are acute respiratory problems and cause high morbidity and high mortality [2,3]. Besides avian species, this disease can affect a wide range of mammalian species, including humans, horses, pigs, dogs, and sea mammals [2]. The combination of two groups of proteins, 16 hemagglutinin (HA) and 9 neuraminidase (NA), produces many subtypes of AIV, but most of them are non-pathogenic or causing mild clinical symptoms [4]. According to pathogenicity, AIV has been divided into two groups: highly pathogenic avian influenza viruses (HPAIV) and low pathogenic avian influenza viruses (LPAIV) [5]. The HPAIV (H5N1 and H7N9) is characterized by severe illness and 100% death in infected birds [6]. HPAI H5N1 was first identified in Bangladesh in February 2007, and still several waves of the outbreak occur every year with high mortality in the poultry sector [7,8]. The prevalence of HPAI H5N1 has a seasonal pattern in Bangladesh, and outbreak waves start registering in late autumn, peaking in spring, and then decrease frequency gradually during the summer season [9]. Now, it is deeply entrenched in almost all countries of Asia, including China, India, Indonesia, and Vietnam [10]. The circulating HPAI H5N1 in Bangladesh was under goose/Guangdong lineage clade 2.2.2 from 2007 to 2010, and clade 2.3.2.1 and clade

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2.3.4.2 were introduced during early 2011, and from 2014 clade 2.3.2.1a is circulating exclusively in the poultry sector [11,12]. LPAI H9N2 is the dominating subtype of LPAIV in Bangladesh and is associated with mild clinical signs in poultry, impacting production performance and a low mortality rate [13]. The co-circulation of HPAI H5N1 and LPAI H9N2 is registered in Bangladesh poultry since 2007, and there is an increased probability that the evolution of viruses could require additional prevention measures [14].

The major propagation factors associated with AIV infections are farm biosecurity practices, flock density, seasonal variation, presence of Anseriformes (duck), and wild or migratory birds [15]. A few studies were conducted in Bangladesh to explore possible risk factors of AIV in backyard chickens and duck farms [16–18]. But there is a lack of risk factors analysis regarding AIV in commercial asymptomatic chickens. Therefore, the study's objective was the surveillance of AIV in asymptomatic commercial chickens and identifying possible risk factors in the two selected regions of Bangladesh.

Materials and Methods

Ethical approval

The study was approved by the Animal Experiment Ethics Committee, Bangladesh Livestock Research Institute (No.: BLRI1002).

Data and sample collection

A cross-sectional study was conducted from July to December 2019 in the Dhamrai sub-district of Dhaka district and Gazipur Sadar sub-district of Gazipur district. A total of 582 (25–26 samples per flock) individual oropharyngeal swab samples were collected in virus transfer media from 23 chicken flocks, including ten broilers, five layers, and eight *Sonali* chicken (cross-breed between Rhode Island Red cocks and Fayoumi hens) flocks. The flocks were classified into three categories according to the number of chickens per flock, counting small (500–1,000 chickens), medium (1,001–1,500), and large (>1,501) flocks. A structured and validated questionnaire was developed and administered to the chicken farmers to record farmers' demographic information, followed by farm demography, biosecurity practices, and management practices. Samples were labeled and placed into an insulated icebox and transferred to the National Reference Laboratory for Avian Influenza, Bangladesh Livestock Research Institute, Dhaka, and stored at –80°C for testing.

Laboratory testing

The magnetic bead-based RNA isolation technology was applied for RNA extraction from collected samples individually using MagMAX™-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems™, San Francisco, CA) in KingFisher™ Flex 96-well robot (Thermo Scientific™, Waltham, MA) according to the manufacturer's protocol. The samples were screened first for *M* gene presence by real-time reverse transcription PCR (rRT-PCR) test using reference primers and probes. Then, *M* gene positive samples were further assessed for H5, H9, N1, N2, N6, and N8 sub-typing using primers and probes by rRT-PCR test. The primers and probes are listed in Table 1.

Statistical analysis

Data from laboratory analysis and questionnaire were recorded and coded in Microsoft Excel 2013 spreadsheet (Microsoft Corporation, Redmond, WA) and exported into STATA-13 (STATA Crop, 4905, Lakeway drive, College Station, TX) for statistical analysis. Descriptive statistics were carried out to calculate the *M* gene's overall prevalence and subtypes of AIV (H5, H9, N1, N2, N6, and N8). The distribution of AIV subtypes was then analyzed according to the study area, type of chicken, flock size, and biosecurity practice. The odds ratios (OR) were calculated to determine the risk factors between individual chickens' positivity with independent variables by multivariable logistic regression. Variables included in the multivariable logistic model were chicken types (layer vs. broiler; layer vs. *Sonali*), flock size (small vs. medium, small vs. large), and biosecurity practice (good vs. moderate and good vs. poor). The results are presented as OR, 95% confidence interval (95% CI), and $p < 0.05$ was used for the statistical significance level.

Results

Overall, 7.73% (45/582) oropharyngeal swabs were found positive for avian influenza (AIV) *M* gene. By locations, the samples were found AIV positive as 7.39% (95% CI: 4.19–11.89; $n = 203$) and 7.92% (95% CI: 5.40–11.11; $n = 379$) of Dhamrai and Gazipur Sadar, respectively (Table 2). In chickens, the *Sonali*-type chickens were significantly ($p = 0.035$) higher and AIV positive than broiler and layer chickens. By flock size, the AIV RNA prevalence was significantly higher in larger flock size (10%; 95% CI: 6.22–15.02; $n = 200$) compared to medium and small flock sizes (4.10%; 95% CI: 1.34–9.31; $n = 122$). The AIV prevalence also found to be significantly higher in poor biosecurity flock (10.66%; 95% CI: 7.26–15.95; $n = 272$) compared to moderate and good biosecurity (3.92%; 95% CI: 1.08–9.74; $n = 102$) practicing flocks (Table 2).

Furthermore, the HA and NA types of 45 AIV positive samples were analyzed (Table 3). There were four combinations of H and N-type that were found, including A/H5N1 (31.11%; $n = 14$), A/H5N2 (28.89%; $n = 13$), A/H9N1 (6.67%; $n = 3$), and A/H9N2 (8.89%; $n = 4$). But there was

Table 1. List of primers and probes used for identification of *Matrix* (*M*) gene of avian influenza and its subtypes.

Target gene	Item	Name	Sequence	Reference
M	Forward	IVA D161M	5′ AGATGAGYCTTCTAACCGAGGTCG 3′	[46]
	Reverse	IVA D162M1	3′ GTCTCTGAACTYCTACAAAAACGT 5′	
		IVA D162M2	3′ GTCTCTGAACTYCTACACAAACGT 5′	
		IVA D162M3	3′ GTCTCTGAACTYCTACAGAAACGT 5′	
		IVA D162M4	3′ GTCTCTGAACTYCTACATAAACGT 5′	
	Probe	IVA-MA	5′-FAM-TCAGGCCCCCTCAAAGCCGA-TAMRA-3′	
H5	Forward	IVA D148H5	5′ AAACAGAGAGGAAATAAGTGGAGTAAAATT 3′	
	Forward	IVA D204f	5′ ATGGCTCCTCGGRAACCC 3′	
	Reverse	IVA D149H5	3′ CGTTAGTACCATCGACCAGATAGAAA 5′	
	Reverse	IVA D205r	3′ GCCTTACCAGAATGTATCACCTYTT 5′	
	Probe	IVA H5a	5′-FAM-TCAACAGTGGCGAGTTCCCTAGCA-TAMRA-3′	
	Probe	IVA D215P	5′-FAM-ATGTGTGACGAATTCMT-MGBNFQ-3′	
H9	Forward	H9	5′ ATGGGGTTTGCTGCC 3′	[47]
	Reverse	H9v2 Rev3	3′ ACGTCYACGTTGTAAACATATA 5′	
	Probe	H9v2 ProbeV4	5′-FAM-TTCTGGGCYATGTCCAATGG-BHQ-1-3′	
N1	Forward	IAV-N1-3-F	5′ AGRCCTTGYTTCTGGGTTGA 3′	
	Reverse	IAV-N1-3-R	3′ ACCAGAACCGGTCTGCCA 5′	
	Probe	IAV-N1-3-FAM	5′FAM-ATYTGGACYAGTGGGAGCAGCAT-BHQ1 3′	
N2	Forward	IAV-N2-1367F	AGT CTG GTG GAC YTC AAA YAG	
	Reverse	IAV-N2-1488R	AAT TGC GAA AGC TTA TAT AGV CAT	
N6	Probe	IAV-N2-1444.1FAM-MGB	FAM-CCA TCA GGC CAT GAG CCT-MGB	
N6	Forward	IAV-N6-10F	5′ AGG GTG AAR ATG AAT CCA AAY CA 3′	
	Forward	IAV-N6-14F	3′ TGA ARA TGA ATC CAA ATC AGA AGA TAA 5′	
	Reverse	IAV-N6-97R	3′ CATCATTCRGACGAYTATCCTAAC 5′	
	Probe	IAV-N6-43FAM	5′-FAM-TGC ATH TCA GCH ACA GGA ATG ACA CTA TC-BHQ1-3′	
N8	Forward	IAV-N8-1296F	5′ TCCATGYTTTTGGGTTGARATGAT 3′	
	Reverse	IAV-N8-1423R	3′ ACCAGYACCGTRCTACCTCG 5′	
	Probe	IAV-N8-1354FAM	5′-FAM-TCHAGYAGCTCCATTGTRATGTGTGGAGT-BHQ1-3′	

8.89% (n = 4) AIV matrix gene-positive samples that could not be identified from this combination, and N6 and N8 subtypes were not detected in any of the samples tested.

The AIV prevalence varied significantly ($p < 0.05$) among the categories of type of chicken, flock size, and biosecurity practices in univariable analysis. These three significant variables were then used for multivariable analysis. The multivariable regression model showed that *Sonali* chickens were 2.0 (95% CI: 0.83–4.83; p = 0.023) times more likely to be AIV positive than layer chickens. In flock size, a larger flock size was 2.6 (95% CI: 0.95–7.11; p = 0.041) times more likely to be AIV prevalent than a smaller flock size. Also, flocks in poor biosecurity practices were found at 2.92 (95% CI: 1.00–8.53; p = 0.042) times more likely to suffer from AIV infection than flocks with practicing good biosecurity (Table 4).

Discussion

AIV has a substantial economic impact on Bangladesh's poultry industry and has been considered a threat to human health [1,14,19]. Several subtypes of AIV have been identified in Bangladesh in various species, such as A/H1N1, A/H1N2, A/H1N3, A/H2N5, A/H3N3, A/H3N6, A/H3N8, A/H4N2, A/H5N1, A/H5N6, A/H7N5, A/H7N9, A/H9N1, A/H9N2, A/H10N7, and A/H11N3 [20–22]. By considering these, the present study was conducted to identify the risk factors of the study of AIVs of commercial chickens in Dhamrai and Gazipur regions of Bangladesh as highly poultry-populated areas.

The study investigated the unexplained epidemiological features of avian influenza in asymptomatic commercial chickens in Bangladesh. Overall, the AIV RNA was

Table 2. Univariate association between potential risk factors and AIV prevalence in poultry farms in Dhamrai and Gazipur, Bangladesh.

Group		No. of flock	Sample tested	*M* gene positive (+) (%)	95% CI	χ^2	*p* value
Type	Broiler	10	250	16 (6.40)	3.70–10.19	6.260	0.035
	Layer	5	125	7 (5.60)	2.28–11.20		
	Sonali	8	207	22 (10.63)	6.78–15.65		
Location	Dhamrai	8	203	15 (7.39)	4.19–11.89	0.845	0.927
	Gazipur	15	379	30 (7.92)	5.40–11.11		
Flock density	Large (>1,500)	8	200	20 (10.00)	6.22–15.02	5.210	0.045
	Medium (1,000–1,500)	10	260	20 (7.69)	4.76–11.63		
	Small (500–1,000)	5	122	5 (4.10)	1.34–9.31		
Biosecurity	Good	4	102	4 (3.92)	1.08–9.74	6.550	0.042
	Moderate	8	208	12 (5.77)	3.02–9.86		
	Poor	11	272	29 (10.66)	7.26–15.95		
Total		23	582	45 (7.73)			

p = Probability value; 95% CI = 95% Confidence interval; χ^2 = Chi-square value; + = Positive sample.

Table 3. Distribution of subtypes of avian influenza prevalence in chickens in Dhamrai and Gazipur, Bangladesh (*n* = 45 AIV positive).

Group		H5N1	H5N2	H9N1	H9N2	HxN1	HxN2	N6	N8	Unknown	Total
	Broiler	6	4	1	2	0	1	0	0	2	16
Type	Layer	2	3	0	1	0	0	0	0	1	7
	Sonali	6	6	2	1	4	2	0	0	1	22
	Large (>1,500)	2	8	2	3	2	1		0	2	20
Flock size	Medium (1,000–1,500)	11	3	1	0	1	2			2	20
	Small (500–1,000)	1	2	0	1	1	0	0	0	0	5
	Good	0	1	1	1	0	1	0	0	0	4
Biosecurity	Moderate	2	4	1	1	1	1	0	0	2	12
	Poor	12	8	1	2	3	1	0	0	2	29
Total		14 (31.11%)	13 (28.89%)	3 (6.67%)	4 (8.89%)	4 (8.89%)	3 (6.67%)	0	0	4 (8.89%)	45

Table 4. Multivariable logistic regression analysis of potential risk factors for avian influenza prevalence in chickens in Dhamrai and Gazipur, Bangladesh.

Group		OR	95% CI	*p* value
	Layer	–	–	–
Type	Broiler	1.15	0.46-2.87	0.001
	Sonali	2	0.83-4.83	0.023
	Small (500–1,000)	–	–	–
Flock size	Medium (1,000–1,500)	1.95	0.71-5.32	0.002
	Large (>1,500)	2.6	0.95-7.11	0.041
	Good	–	–	–
Biosecurity	Moderate	1.5	0.47-4.77	0.011
	Poor	2.92	1.00-8.53	0.042

p = Probability value; 95% CI = 95% Confidence interval; OR = Odd ratio.

found in 7.73% of the chickens with no visible abnormalities. The results of the study indicated AIV is prevalent in commercial chickens in Bangladesh. Estoepangestie et al. [23] isolated highly pathogenic avian influenza virus (HPAI) A/H5N1 clade 2.3.2.1 from an asymptomatic duck in Indonesia in 2019, and ducks can act as a potential spreader of AIV when shedding virus. The report agreed with the study that indicated asymptomatic birds could carry A/H5N1.

HPAI H5N1 is a significant cause of poultry mortality and human infections worldwide [24]. The world faced a century ago the Spanish flu in 1919 (H1N1) with 50 million mortalities and sickness of one-fourth of the world's total population [25]. Since the first identification of HPAI H5N1 in Bangladesh in 2007, several AIV outbreaks have occurred every year [8,26]. The study identified 31.11%

samples out of total AIV positive samples were A/H5N1 positive. Surveillance conducted by the National Reference Laboratory for Avian Influenza Bangladesh on HPAI H5N1 reported 323 cases till 2009 [27], and there were 44% cases from small commercial farms [28]. The live bird market surveillance also reported that 47.4% samples were positive for AIV where 21.26% had HPAI H5N1 and distribution on a different type of chickens was including 36.3% in chickens (broiler 47.4%, *Sonali* 31.7%, and Deshi 39.4%), and 18.7% in waterfowl (duck 19.3% and geese 16.7%) [29]. Negovetich et al. [30] reported that 23% of the live bird markets were positive for AIV, where 94% of infections were low pathogenic avian influenza (LPAI). After the first identification of HPAI in Bangladesh, both A/H5N1 and A/H5N2 were co-circulating in the poultry farms, environments, and LMBs [31]. HPAI H5N1 was also identified in various species other than chickens in Bangladesh like crow, bats, monkey, waterfowl, goose, quails, and pigeons [26,32,33].

A/H9N1 and A/H9N2 are a low pathogenic avian influenza bearing low mortality with the continuous loss of egg and meat production in the poultry industry and significant economic losses [34,35]. Here, 6.67% of the samples were found to be A/H9N1 positive and 8.89% to be A/H9N2 positive. The previously published report identified 16.5% of A/H9N2 in selected poultry farms in Bangladesh [30]. The LPAI causes 90% of morbidity in chickens, with continuous production loss occurring in the poultry industry in Bangladesh [14].

The study was identified as a significant risk factor of increasing the prevalence of AIV in the flocks, including the type of chicken, flock size, and biosecurity practices. *Sonali* chickens were identified as potentially vulnerable to the infection with AIV compared to broiler and layer chickens. Kim et al. [29] demonstrated that *Sonali* chickens harbor a significant infection load. The husbandry practice of *Sonali* chickens is under poor biosecurity and larger flock size [36,37]. The breeding policy was also traditional and non-systematic [36]. Other avian viral diseases like infectious bronchitis, avian metapneumovirus, and reticuloendotheliosis virus reported a higher prevalence in selected areas of Bangladesh [38–40]. Therefore, *Sonali* acts as a potential spreader and harbors AIV infection compared to the other poultry flocks.

Flock density is also a significant risk factor for rapid transmission and higher prevailing of AIV. The AIV can be transmitted from bird to bird by direct contact with secretions, feces, and aerosol droplets [41]. So higher flock density can increase the spread of AIV rapidly from bird to bird [15].

Tiensin et al. [15] reported that high flock density is a potential risk factor to transmit AIV, and they identified AIV risk to be 0.98 times and 1.02 times higher in backyard chickens and fighting cock chickens in Thailand, respectively. Therefore, there is a strong association between flock density and AIV infection in birds.

Poor biosecurity practice is a significant risk factor for the higher prevalence of AIV [42]. Waziri et al. [43] demonstrated that chickens reared in poor biosecurity practices were three times more vulnerable to be spreaders of AIV compared to good biosecurity practicing farms, which is in accordance with the current findings. Osmani et al. [16] conducted a case control study and reported an increased number of farm staffs (OR: 5.2), weekly visit of veterinarians (OR: 3.0), and roaming of village chickens around the farm (OR: 0.6) were the major biosecurity risk factors in commercial poultry farms in Bangladesh. This study agreed with our biosecurity investigation. Large-scale poultry farms usually maintain good biosecurity practices due to standard operation and commercial establishment, but it is relatively poor in small-scale farms. As a result, maintaining good biosecurity is sometimes compromised in small-scale poultry farms and poses a higher risk of AIV [22,44,45]. So, it is obligatory to practice good farm biosecurity to control flocks from AIV.

However, A/H5N1 and A/H9N2 are prevalent and sometimes co-circulating in different chicken types in Bangladesh.

Conclusion

The co-circulation of HPAI H5N1and LPAI H9N2 is reported in the commercial poultry industry since the first identification of HPAI H5N1in Bangladesh. Birds carrying A/H5N1 without showing clinical symptoms is a big concern for the poultry and public health sectors. Poor farm biosecurity practices, large flock density, and *Sonali* chickens are identified as potential risk factors related with AIVs infection in commercial chickens in Bangladesh. Enhancing biosecurity and proper vaccination practices against HPAI H5N1and LPAI H9N2 could be an effective AIVs control measure in Bangladesh. Further study of amino acids arrangement of the cleavage site of the HA molecule for the determination of HPAIV H5N1 and LPAIV H9N2 is suggested.

List of abbreviations

95% CI: 95% CIs, AIV: Avian influenza virus, HA: Hemagglutinin, HPAIV: Highly pathogenic avian influenza virus, LPAI: Low pathogenic avian influenza virus, NA: Neuraminidase, OR: Odds ratio, RNA: Ribonucleic acid, rRT-PCR: Real-time reverse transcription PCR, LBM: Live Bird Market.

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Conflict of interest

The authors declare no conflicts of interest.

Authors' contributions

MZ Ali conceived, designed the study, analyzed the data, and wrote the article. M Hasan conducted surveillance and laboratory analysis. M Giasuddin reviewed and finalized the article. All the authors read the final version and approved for publication of the article.

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