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Exploring Risk Factors for Avian Influenza A Virus in Poultry: A Cross-Sectional Study in Dhaka Division, Bangladesh

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Abstract

AVIAN INFLUENZA VIRUS (AIV) significantly affects both commercial and backyard poultry by causing high mortality rates or diminishing egg production, while also posing public health risks. We conducted a cross-sectional study in live bird markets (LBMs), as well as in backyard and commercial poultry farms located in Dhaka (Savar), Gazipur (Gazipur Sadar), and Tangail (Mirzapur) in Bangladesh. Seventy-seven swab samples were obtained from backyard chickens (tracheal swabs=20), commercial chickens (tracheal swabs=34), and environmental swabs from LBMs (n=23) between January and February 2020. Using real-time rRT-PCR, we detected an overall prevalence 22.8% (17/77) for AIV, with specific rates of 16.67% (95% CI: 5.64-34.72) in Tangail, 30.43% (95% CI: 13.21-52.92) in Savar, and 20.83% (95% CI: 7.13-42.15) in Gazipur. We conducted a multivariate logistic regression analysis to identify potential risk variables related with AIV and determined that sample sources, management of sick and dead chickens, and housing systems significantly contribute to AIV infections. The identified risk factors for AIV infection included sampling sources (commercial chicken - AOR: 1.16; 95% CI: 0.2859-4.7615; LBMs - AOR: 2.04; 95% CI: 0.5840-7.13), management of sick chickens (sold - AOR: 2.04; 95% CI: 0.58-7.13, eaten - AOR: 1.16; 95% CI: 0.28-4.76), management of dead chickens (throw in bushes - AOR: 1.60; 95% CI: 0.52-4.89), and housing systems (caged- AOR: 1.29; 95% CI: 0.20-8.30), all of which were statistically significant ($p<0.05$). In conclusion, AIV is prevalent in various farming practices and LBMs. Consequently, regular monitoring of AIV is strongly advised to assess the current status of AIV in Bangladesh.

Keywords: Avian influenza, LBM, backyard chicken, Bangladesh, Poultry.

Introduction

Influenza A viruses are currently circulating in both animals and humans globally; vigilant surveillance is crucial for monitoring the real-time situation. Avian influenza viruses (AIVs) replicate in the respiratory and/or digestive tracts of infected birds [1]. The Type A influenza virus, a negative-sense, single-stranded, segmented RNA virus from the Orthomyxoviridae family, is responsible for avian influenza in birds [2]. AIVs are spontaneously transmitted from wild birds to domesticated chickens at points of coexistence. Typically, domestic birds infected with the AIV virus transmit it to humans. We can determine the incidence of these diseases, enhance our comprehension of their transmission at the human-animal interface, and formulate measures to mitigate the risk of such transmission by dynamic longitudinal surveillance of both domestic and wild birds [3]. Alongside avian species, other mammalian species, such as dogs, horses, pigs, and warm-blooded marine creatures, are vulnerable to this disease [4]. The 16 hemagglutinin (HA) and 9 neuraminidase (NA) protein combinations collaborate to create many avian influenza virus (AIV) subtypes, most of which

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are either non-pathogenic or exhibit mild clinical symptoms [5].

Bangladeshi poultry populations are endemic for both highly pathogenic (H5N1) and low pathogenicity (H9N2) avian influenza A viruses [1]. Severe disease and complete mortality in infected avians are characteristic features of HPAIV (H5N1 and H7N9) [6]. In addition to their harmful effects on poultry productivity, these viruses have sporadically resulted in influenza cases in humans [7,8]. Their persistent proliferation in chickens presents a significant threat to the health of humans and animals globally due to the potential emergence of novel reassortant variants among them or in conjunction with other viral subtypes [9,10].

Highly Pathogenic Avian Influenza Virus H5N1 was initially identified in Bangladesh in February 2007, and annually, the H5N1 outbreak results in significant mortality within the poultry industry [1,11]. A multitude of mutations facilitated the virus's transition from genetic clade 2.2.2 to clade 2.3.2.1a. Since 2013, clade 2.3.2.1a has predominated over other clades [12]. In 2016, an alternative clade (2.3.4.4) of the H5N6 HA gene was found [1]. AIV outbreaks predominantly occur seasonally, with a higher prevalence in winter and spring compared to other seasons. Therefore, it is essential to consistently observe the progression of AIVs in domestic chickens. Bangladesh, a highly populated nation with 1,077 individuals per square kilometre has 90% of rural families engaged in poultry farming, resulting in close closeness between avians and humans [13,14]. In this environment of close interaction between people and poultry (chickens and ducks), co-infection with various avian influenza viruses (AIVs) may occur, increasing the likelihood of novel AIV strains emerging.

Many Asian poultry industries are based on live bird marketplaces, or LBMs. Daily introduction of birds from diverse regions and types into LBMs promotes local transmission of several virus subtypes [15]. In Bangladesh, surveys and routine surveillance have identified avian influenza A viruses (AIVs) in backyards, LBMs, and commercial [16]. A robust LBM AIV prevalence evaluation is lacking, despite its importance to understanding AIV epidemiology and enhancing monitoring design [17].

In addition to susceptibility, different chicken types can be farmed in distinct farming methods and exchanged through different value chains, which are the operations firms conduct to deliver products to clients. They may be exposed to various diseases and loads [11,18]. However, the percentage of AIV-positive samples is usually estimated or divided by chicken type.

The sources from which traders receive poultry and the time they spend in LBMs affect the probability of virus introduction and amplification in LBMs, depending on whether traders are wholesalers or retailers [19]. However, such data is often missed or poorly reported.

The study addressed these issues by conducting a cross-sectional survey in Bangladesh's three largest cities—Dhaka, Gazipur, and Tangail. We estimated the AIV epidemic in LBMs, commercial chicken farms, and backyard native hens, then we identified risk factors.

Method and Materials

Sample collection

Tracheal swab samples from chickens and environmental samples from live bird markets (cages, feed, drinking water, slaughtering surfaces and utensils, slaughtering by-products, shop floors, or trash bins) were collected in viral transport media (VTM) contained within 15ml Falcon tubes. We amalgamated six to seven samples from each business for collective examination. Seventy-seven swab samples were collected from hens from commercial farms (n=34), live bird markets (n=23), and backyard flocks (n=20). Samples were gathered from three poultry-populated regions in Bangladesh: Dhaka (Savar), Gazipur (Gazipur Sadar), and Tangail (Mirzapur), as illustrated in Figure 1. The samples were transported in an ice box to the laboratory and stored in the laboratory refrigerator at -80°C. A pretested and structured questionnaire was utilised to gather potential parameters associated with AIV infection.

RNA Extraction and qRT-PCR

The swab samples were homogenized by vortexing for two to three seconds before RNA extraction. The magnetic bead-based RNA isolation method was used to extract RNA from each collected sample individually using a MagMAX™-96 AI/ND Viral RNA Isolation Kit (Applied Biosystems™, USA) in a KingFisher™ Flex 96 well robot (Thermo Scientific™, USA) by manufacturer instructions. The purified RNA was ready for use in qRT-PCR and other enzymatic reaction. A set of reference primers and probe was used for amplifying the matrix gene of AIV (Table 1). The cycle threshold (Ct) value minimum 38 was considered as AIV positive.

Statistical analysis

The Microsoft Excel expectations 2013 spreadsheet (Microsoft Organization, Redmond, WA) was used to record and code the data before being transmitted to STATA-13 (STATA Crop, 4905, Lakeway Drive, College Station, TX) for factual analysis. To evaluate the overall prevalence of AIV, descriptive statistics were used. Next, the distribution of AIV was examined in relation to the study region, chicken varieties, feed habit, farmer's level of education and age using univariable logistic regression. After that, significant variables were considered for

multivariable logistic regression analysis to compute the adjusted odds ratios (AOR) to identify the potential risk factors between each chicken's positive with respect to independent variables. The outcomes are displayed as AOR, 95% confidence interval (95% CI), and the statistical significance level was set at $p \leq 0.05$.

Results

Fifty-four chicken tracheal swab samples and 23 environmental samples from a total of 77 samples were examined. Overall, 17 (22.08%) swabs out of 77 samples tested positive for the M gene of avian influenza (AIV). Samples were found to be AIV positive by locations as 16.67% (95 % CI: 5.64-34.72; n=30), 30.43% (95 % CI: 13.21-52.92; n=23) and 20.83% (95 % CI: 7.13-42.15; n=24) of Tangail, Savar and Gazipur, respectively. The prevalence of AIV in LBMs were 30.43% (95 % CI: 13.21-52.92; n=23) and in backyard chickens 20% (95 % CI: 5.73-43.66; n=20) and in commercial chickens 17.65% (95 % CI: 6-34.53; n=34). Within sample types, the prevalence of AIV was 37.5% (95 % CI: 15.2-64.5; n=16) in the broiler, 22.22% (95 % CI: 6.4-47.6; n=18) in the layer, 20% (95 % CI: 5.7-43.6; n=20) in native and 13.04% (95 % CI: 2.7-33.5; n=23) in environment. Among educational qualifications, graduate farmers were less likely to be AIV positive than SSC and HSC pass farmers. In the study, the prevalence of AIV in LBMs was 36.37% (95 % CI: 17.2-59.34; n=22) in SSC pass farmers, 19.35% (95 % CI: 7.4-37.4; n=31) in HSC pass farmers and 12.5% (95 % CI: 2.6-32.3; n=24) in graduated farmers. Between dead chicken, 17.65% (95 % CI: 6.76-34.53; n=34) AIV-positive chickens were buried and 25.58% (95 % CI: 13.52-41.17; n=43) AIV-positive chickens were thrown. Prevalence of AIV also got to be significantly higher intake in ready feed (22.8%; 95 % CI: 12.74-35.84; n=7) compared to homemade (20.0%; 95 % CI: 0.51-71.64; n=5) and scavenging feed habit (20.0%; 95 % CI: 4.33-48.09; n=15). An infection rate was 15.38% (95% CI: 1.92-45.45; n=13) in floor living chickens and 19.05% (95% CI: 5.45-41.91; n=21) in caged chickens (Table 2).

The categories of sample source, management of sick chicken, management of dead chicken, and housing system in chickens showed significant ($p \leq 0.05$) variations in AIV prevalence when analyzed with univariate logistic regression analysis (Table 3). Afterwards, multivariable logistic regression analysis was performed using these four significant factors to determining the potential risk factors. The results of the multivariable regression model indicated that the LBM samples were 2.04 (95% CI: 0.58-7.13) times higher AIV positive than backyard chickens. In case of management of sick chickens, sold sick chickens were 2.04 (95% CI: 0.58-7.13) times more likely to be AIV positive than eat the sick chickens. In case of management of dead chickens, thrown away dead chickens were 1.60 (95% CI: 0.52-4.89) times more likely to be AIV positive than bury chickens. Also, caged chickens were found at 1.29 (95% CI: 0.20-8.30) times more likely to suffer AIV infection than floor chicken (Table 3).

Discussion

Avian influenza viruses (AIVs) are the primary cause of widespread disease in domestic birds, resulting in increased morbidity and mortality rates, as well as substantial economic losses worldwide. AIV presents a public health concern and exerts a considerable economic influence on Bangladesh's chicken industry [21]. The H5 subtype of highly pathogenic avian influenza viruses (HPAIV) and the H9 subtype of low pathogenic avian influenza viruses (LPAIV) have become the most prevalent avian influenza viruses (AIV) in poultry, leading to frequent disease outbreaks across various Asian countries [1]. This study aimed to assess the prevalence of AIVs in the commercial live bird market and backyard chickens in the Gazipur, Tangail, and Savar regions of Bangladesh. This investigation evaluated 17 samples from a total of 77 tracheal swab samples. AIV infections were detected in 22.08% of the study population following multiplex real-time RT-PCR analysis of all swab samples. The incidence of AIV virus was highest in live bird market hens due to their origin from various regions of the wet markets. In comparison to other AIV-endemic countries, Egypt, Vietnam and Bangladesh exhibit a greater prevalence of LBM levels of the AIV virus [15]. The prevalence of Avian Influenza Virus (AIV) in backyard hens was 20%, attributed to inadequate hygiene, insufficient biosecurity measures, and lower vaccination rates. The p-value for the sample source was 0.04, falling within the range ($p \leq 0.05$), indicating a substantial connection between AIV infection and the sample source.

The study revealed varying infection rates across three regions of Bangladesh: Savar, Mirzapur, and Gazipur. In the Savar region, 7 out of 23 samples tested positive for avian influenza viruses, resulting in an infection rate of 30.43%. In the second region, Mirzapur, 5 out of 30 samples tested positive for avian influenza viruses, resulting in an infection rate of 16.67%. In the third region of Gazipur, 4 out of 24 samples tested positive for avian influenza viruses, resulting in an infection rate of 16.67%. The infection rates of AIVs in Savar are markedly greater than those in Mirzapur and Gazipur. Environmental variables contribute to the prevalence of AIVs [22].

This study examined the association between the development of AIV and feeding habits as a criterion. The total percentage of positive samples is 22.08%; 22% of chickens use commercial feed, 20% consume homemade food, and 25% forage for food.

The p value (p=0.24) suggested an absence of a significant connection between avian influenza virus infection and feeding habits. Toro et al. [23] demonstrated that feed does not affect AIV transmission, but feeding management does.

The study identified that the prevalence of AIV was higher (19.05%) in caged chickens compared to those on the floor. Caged birds are reared in denser conditions, which may facilitate the transmission of infection [24]. The incidence of AIV virus is greater in discarded dead hens (25.58%) compared to burial (17.65%). It was obvious that discarding deceased or ill birds suspected of avian influenza virus (AIV) increases the likelihood of AIV transmission through direct contact with predators, contravening farm biosecurity protocols [25].

Conclusion

The overall results suggested that the outbreak of avian influenza virus (AIV) persisted in our selected locations in Bangladesh. The study indicates that the prevalence of AIV was highest in Savar and least in Tangail. The highest frequency of AIV was observed in broiler chickens, whereas the lowest prevalence was noted in layer chickens. The incidence of AIV was greatest in live bird markets and least in commercial poultry farms. The disposal of deceased chickens and the sale of diseased chicks were identified as potential risk factors for the prevalence of AIV in that region. Implementing appropriate biosecurity protocols, such as immunisation, sanitising poultry enclosures, and disposing of deceased birds in designated, restricted areas, can mitigate the incidence of disease. Regular training for farmers is essential to avoid AIV transmission. To encourage public engagement, it is essential to establish priorities for the development and implementation of educational efforts about avian influenza and to evaluate their effectiveness. Routine surveillance can be beneficial for the early identification and response to diseases.

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Conflicted interested

None

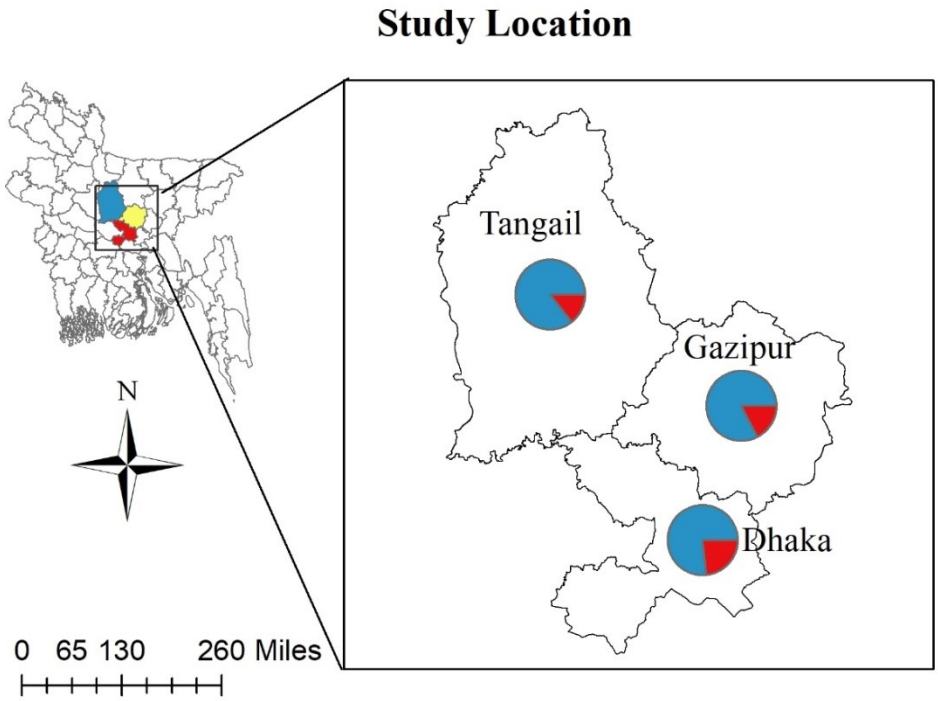

Fig. 1. Study location with AIV prevalence (red colour in pie charts).

TABLE 1. List of primers and probes used for identification of Matrix (M) gene of avian influenza and subtype identification

Target gene	Item	Name	Sequence	Reference
Matrix	Forward	IVA D161M	5' AGATGAGYCTTCTAACCGAGGTCG 3'	[20]
	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'	

TABLE 2. Univariate association between potential risk factors and AIV prevalence

Variables	Categories	No. Tested	M gene positive (%)	95% CI	*p* value
Sample source	LBM	23	7(30.43%)	13.21-52.92	0.05
	Backyard	20	4(20.00%)	5.73-43.66	
	Commercial	34	6(17.65%)	6-34.53	
Education	SSC	22	8(36.37%)	17.2-59.34	0.14
	HSC	31	6(19.35%)	7.4-37.4	
	Graduate	24	3(12.5%)	2.6-32.3	
Sampling location	Tangail	30	5(16.67%)	5.64-34.72	0.634
	Savar	23	7(30.43%)	13.21-52.92	
	Gazipur	24	5(20.83%)	7.13-42.15	
Feed habit	Ready feed	57	13(22.8%)	12.74-35.84	0.24
	Home made	5	1(20.0%)	0.51-71.64	
	Scavenging	15	3(20.0%)	4.33-48.09	
Management of sick chicken	Eat	20	4(20.0%)	5.73-43.66	0.043
	Medication	34	6(17.65%)	6.76-34.53	
	Sold	23	7(30.43%)	13.21-52.92	
Management of dead chicken	Bury	34	6(17.65%)	6.76-34.53	0.031
	Throw	43	11(25.58%)	13.52-41.17	
Sample type	Broiler	16	6(37.5%)	15.2-64.5	0.07
	Native	20	4(20.0%)	5.7-43.6	
	Layer	18	4(22.22%)	6.4-47.6	
	Environment	23	3(13.04%)	2.7-33.5	
Housing system	Floor	13	2(15.38%)	1.92-45.45	0.05
	Caged	21	4(19.05%)	5.45-41.91	

p = Probability value; 95% CI = 95% Confidence interval

TABLE 3. Multivariable logistic regression analysis of potential risk factors for avian influenza prevalence in chickens.

Variables	Categories	AOR	p value
Sample sources	Commercial	Ref.	-
	Backyard	1.16 (0.28-4.76)	0.04
	LBM	2.04 (0.58-7.13)	0.05
Management of sick chicken	Medication	Ref.	-
	Eat	1.16 (0.28-4.76)	0.05
	Sold	2.04 (0.58-7.13)	0.04
Management of dead chicken	Bury	Ref.	-
	Throw	1.60 (0.52-4.89)	0.03
Housing system	Floor	Ref.	-
	Caged	1.29 (0.20-8.30)	0.04

p = probability value; AOR = adjusted odd ratio

References

1. Ali, M.Z., Hasan, M. and Giasuddin, M., Potential risk factors of avian influenza virus infection in asymptomatic commercial chicken flocks in selected areas of Bangladesh during 2019. *J. Adv. Vet. Anim. Res.*, **8**(1), 51. (2021a)
2. VanDalen, K.K., Franklin, A.B., Mooers, N.L., Sullivan, H.J. and Shriner, S.A., Shedding light on avian influenza H4N6 infection in mallards: modes of transmission and implications for surveillance. *PLoS One*, **5**(9), p.e12851, (2010).
3. Kwon, J.H., Lee, D.H., Criado, M.F., Killmaster, L., Ali, M.Z., Giasuddin, M., Samad, M.A., Karim, M.R., Hasan, M., Brum, E. and Nasrin, T., Genetic evolution and transmission dynamics of clade 2.3. 2.1 a highly pathogenic avian influenza A/H5N1 viruses in Bangladesh. *Virus Evol.*, **6**(2), p.veaa046, (2020).
4. Webster, R.G., Bean, W.J., Gorman, O.T., Chambers, T.M. and Kawaoka, Y., Evolution and ecology of influenza A viruses. *Microb. Rev.*, **56**(1), 152-179(1992).
5. Alexander, D.J., Summary of avian influenza activity in Europe, Asia, Africa, and Australasia, 2002–2006. *Avian Dis.,* **51**(s1), 161-166 (2007).
6. Swayne, D.E. and Suarez, D.L., Highly pathogenic avian influenza. *Rev. Sci. Tech. - Off. Int. Epizoot.,* **19**(2), 463-475, (2000)**.**
7. Negovetich, N.J., Feeroz, M.M., Jones-Engel, L., Walker, D., Alam, S.R., Hasan, K., Seiler, P., Ferguson, A., Friedman, K., Barman, S. and Franks, J., Live bird markets of Bangladesh: H9N2 viruses and the near absence of highly pathogenic H5N1 influenza. *PloS one*, **6**(4), p.e19311, (2011).
8. Bhuiyan, Z.A., Ali, M.Z., Moula, M.M., Giasuddin, M. and Khan, Z.U.M., Prevalence and molecular characterization of infectious bronchitis virus isolated from chicken in Bangladesh. *Vet. World*, **12**(6), 909, (2019a.).
9. Monne, I., Yamage, M., Dauphin, G., Claes, F., Ahmed, G., Giasuddin, M., Salviato, A., Ormelli, S., Bonfante, F., Schivo, A. and Cattoli, G., Reassortant avian influenza A (H5N1) viruses with H9N2-PB1 gene in poultry, Bangladesh. *Emerg. Infect. Dis.*, **19**(10), 1630(2013).
10. Ali, M.Z., Shaon, M.T.W., Moula, M.M., Bary, M.A., Sabuj, A.A.M., Khaled, S.A., Bhuiyan, Z.A. and Giasuddin, M., First report on the seroprevalence of avian encephalomyelitis virus antibody in Sonali (cross-bred) chickens in Bogura, Bangladesh. *J. Adv. Vet. Anim. Res.*, **8**(1), 78 (2021b).
11. Biswas, P.K., Christensen, J.P., Ahmed, S.S., Barua, H., Das, A., Rahman, M.H., Giasuddin, M., Hannan, A.S., Habib, M.A., Ahad, A. and Rahman, A.S., Avian influenza outbreaks in chickens, Bangladesh. *Emerg. Inf. Dis.*, **14**(12), 1909(2008).
12. Nooruzzaman, M., Haque, M.E., Chowdhury, E.H. and Islam, M.R., Pathology of clade 2.3. 2.1 avian influenza virus (H5N1) infection in quails and ducks in Bangladesh. *Avian Pathol.*, **48**(1), 73-79, (2019).
13. Bhuiyan, Z.A., Ali, M.Z., Moula, M.M., Bary, M.A., Arefin, N., Giasuddin, M. and Khan, Z.U.M., Seroprevalence of major avian respiratory diseases in broiler and sonali chicken in selected areas of Bangladesh. *J. Adva. Vet. Anim. Res.,* **6**(4), 561 (2019b).
14. Ali, M.Z. and Hasan, B., Follow up of maternally derived antibodies titer against economically important viral diseases of chicken. *Poult. Sci. J.,* **6**(2), 149-154(2018).
15. Nguyen, D.C., Uyeki, T.M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., Smith, C., Rowe, T., Lu, X., Hall, H. and Xu, X., Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1, from poultry in live bird markets in Hanoi, Vietnam, in 2001. *J. Virol.*, **79**(7), 4201-4212 (2005).
16. Sayeed, M.A., Smallwood, C., Imam, T., Mahmud, R., Hasan, R.B., Hasan, M., Anwer, M.S., Rashid, M.H. and Hoque, M.A., Assessment of hygienic conditions of live bird markets on avian influenza in Chittagong metro, Bangladesh. *Preven. Vet. Med.,* **142**, 7-15(2017).
17. Ali, M.Z., Sana, S., Sheikh, A.A. and Maheen, Z., Molecular characterization of toxigenic aspergillus flavus isolated from sick broiler lungs and risk factors analysis. *Pak. Vet. J.*, **42**(2), 194-200(2022).
18. Ali, M.Z. and Islam, M.M., Characterization of β-lactamase and quinolone resistant Clostridium perfringens recovered from broiler chickens with necrotic enteritis in Bangladesh. *Iran. J. Vet. Res.*, **22**(1), 48 (2021).
19. Fournié, G., Guitian, F.J., Mangtani, P. and Ghani, A.C., Impact of the implementation of rest days in live bird markets on the dynamics of H5N1 highly pathogenic avian influenza. *J. R. Soc. Interface*, **8**(61), 1079-1089, (2011).
20. Heine, H.G., Foord, A.J., Wang, J., Valdeter, S., Walker, S., Morrissy, C., Wong, F.Y. and Meehan, B., Detection of highly pathogenic zoonotic influenza virus H5N6 by reverse-transcriptase quantitative polymerase chain reaction. *Virol. J.*, **12**, 1-4 (2015).
21. Parvin, R., Begum, J.A., Nooruzzaman, M., Chowdhury, E.H., Islam, M.R. and Vahlenkamp, T.W., Review analysis and impact of co-circulating H5N1 and H9N2 avian influenza viruses in Bangladesh. *Epidemiol. Inf.*, **146**(10), 1259-1266 (2018).
22. Bo, H., Zhang, Y., Dong, L.B., Dong, J., Li, X.Y., Zhao, X., Li, Z., Shu, Y.L. and Wang, D.Y., Distribution of avian influenza viruses according to environmental surveillance during 2014–2018, China. *Inf. Dis. Poverty*, **10**(1), 60 (2021).
23. Toro, H., Van Santen, V.L. and Breedlove, C., Inactivation of avian influenza virus in nonpelleted chicken feed. *Avian Dis.*, **60**(4), 846-849 (2016).
24. Contreras, A., Gómez-Martín, A., Paterna, A., Tatay-Dualde, J., Prats-Van Der Ham, M., Corrales, J.C., De La Fe, C. and Sánchez, A., Epidemiological role of birds in the transmission and maintenance of zoonoses. *Rev. Sci. Tech.*, **35**(3), 845-862(2016).
25. FAO, V.C., Prevention and control of avian flu in small scale poultry. *A guide for veterinary paraprofessionals in Cambodia. Romes: FAO*, 1-36, (2005).