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Potential risk zones and climatic factors influencing the occurrence and persistence of avian influenza viruses in the environment of live bird markets in Bangladesh

Ariful Islam [a,b,*], Emama Amin [a,c], Sarah Munro [a], Mohammad Enayet Hossain [d], Shariful Islam [a,c], Mohammad Mahmudul Hassan [e], Abdullah Al Mamun [a,c], Mohammed Abdus Samad [f], Tahmina Shirin [c], Mohammed Ziaur Rahman [d], Jonathan H. Epstein [a]

[a] *EcoHealth Alliance, New York, NY 10018, USA*
[b] *School of Life and Environmental Sciences, Deakin University, Geelong, Victoria, Australia*
[c] *Institute of Epidemiology, Disease Control and Research (IEDCR), Dhaka 1212, Bangladesh*
[d] *One Health Laboratory, International Centre for Diarrheal Diseases Research, Bangladesh (ICDDR), Bangladesh*
[e] *Queensland Alliance for One Health Sciences, School of Veterinary Science, University of Queensland, QLD 4343, Australia.*
[f] *National Reference Laboratory for Avian Influenza, Bangladesh Livestock Research Institute (BLRI), Savar, Bangladesh*

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ABSTRACT

Live bird markets (LBMs) are critical for poultry trade in many developing countries that are regarded as hotspots for the prevalence and contamination of avian influenza viruses (AIV). Therefore, we conducted weekly longitudinal environmental surveillance in LBMs to determine annual cyclic patterns of AIV subtypes, environmental risk zones, and the role of climatic factors on the AIV presence and persistence in the environment of LBM in Bangladesh. From January 2018 to March 2020, we collected weekly fecal and offal swab samples from each LBM and tested using rRT-PCR for the M gene and subtyped for H5, H7, and H9. We used Generalized Estimating Equations (GEE) approaches to account for repeated observations over time to correlate the AIV prevalence and potential risk factors and the negative binomial and Poisson model to investigate the role of climatic factors on environmental contamination of AIV at the LBM. Over the study period, 37.8% of samples tested AIV positive, 18.8% for A/H5, and A/H9 was, for 15.4%. We found the circulation of H5, H9, and co-circulation of H5 and H9 in the environmental surfaces year-round. The Generalized Estimating Equations (GEE) model reveals a distinct seasonal pattern in transmitting AIV and H5. Specifically, certain summer months exhibited a substantial reduction of risk up to 70–90% and 93–94% for AIV and H5 contamination, respectively. The slaughtering zone showed a significantly higher risk of contamination with H5, with a three-fold increase in risk compared to bird-holding zones. From the negative binomial model, we found that climatic factors like temperature and relative humidity were also significantly associated with weekly AIV circulation. An increase in temperature and relative humidity decreases the risk of AIV circulation. Our study underscores the significance of longitudinal environmental surveillance for identifying potential risk zones to detect H5 and H9 virus co-circulation and seasonal transmission, as well as the imperative for immediate interventions to reduce AIV at LBMs in Bangladesh. We recommend adopting a One Health approach to integrated AIV surveillance across animal, human, and environmental interfaces in order to prevent the epidemic and pandemic of AIV.

1. Introduction

Live bird markets (LBMs) are the primary gateway for poultry marketing throughout Bangladesh and serve as cornerstones for viral accumulation, amplification, and the transmission of many infectious diseases, including the avian influenza virus (AIV) [1,2]. In many countries, LBMs have been linked to the dynamics of AIV transmission, dispersal, and sustained circulation, allowing the reassortment of various virus strains. According to previous studies, LBMs in Bangladesh have a higher prevalence of influenza A/H5N1 and A/H9N2 viruses and

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[*] Corresponding author at: EcoHealth Alliance, New York, NY 10018, USA.
E-mail address: arif@ecohealthalliance.org (A. Islam).

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may be a significant source of bird-to-human transmission [3–5]. The possibility of the highly pathogenic avian influenza (HPAI) A/H5N1 causing severe morbidity and death in birds is a worldwide concern [6]. H5N1 spreads swiftly among domestic chickens and produces widespread outbreaks, causing significant losses for the global poultry sector [7]. From 2007 to 2022, Bangladesh reported 585 HPAI H5N1 outbreaks in poultry and wild birds, with commercial poultry farms accounting for 90% of the cases [8]. Human infections may result from direct and indirect contact, such as exposure to an H5N1-infected environment or exposure to live poultry markets [9–11]. The co-infection of HPAI H5N1 and LPAI viruses, particularly H9N2, is also a significant concern [12]. The reassortment of these LPAI viruses with HPAI H5N1 might produce new influenza viruses that can infect humans despite the interspecies barrier [13]. Bangladesh has documented eight human cases of H5N1, with one death and three mild human cases of H9N2, and the virus continues to cause occasional poultry outbreaks in different regions of the country [14]. In urban, peri-urban, and rural Bangladesh, there are numerous LBMs where multiple poultry species are kept in the same cage, supplied by middlemen, and obtained from various backyard and commercial poultry farms [15]. Additionally, LBM practices such as selling multiple types of poultry in the same stalls, a lack of proper sanitary precautions, and transit of infected substances could facilitate the viral spread into diverse species and poultry production systems [16]. Several components of a poultry production system can be subject to biosecurity regulations, such as personnel and visitor restrictions, limiting contact between chickens and other animals, proper shed sanitation, equipment, vehicle disinfection, and complete water treatment [17]. If an infectious disease is introduced and established, guidelines are in place to minimize its spread. Biosecurity practices diminish incrementally as poultry passes through numerous chains to reach LBMs, at which point various infectious diseases such as AIV may readily circulate from bird to bird, bird to human, and bird to wild bird [18]. Poor hygiene and biosecurity practices are common among LBMs, making them suitable for AIV infection and transmission [19]. As a result, LBMs may act as hot spots for virus evolution and the emergence of new strains, which is particularly concerning given the role these markets play in providing protein to the population [20]. Specifically, LBM with wholesale was more contaminated with H5 than only the retail market [3]. LBM workers do not have proper knowledge about AIV and do not follow biosecurity measures properly. Nearly half of the LBM workers do not wash their hands with soap and clean the stall and cage daily [21]. There was limited precaution among the workers when handling sick poultry.

LBMs typically provide foraging grounds for peri-domestic birds, such as house crows, sparrows, and starlings, which may facilitate additional opportunities for cross-species transmission of AIV. House crows (*Corvus splendens*) in Bangladesh have previously been found positive for HPAI H5N1 and have thus been implicated in transmitting AIV [22,23]. HPAI H5N1 outbreak in Bangladesh proved that the virus is rooted and distributed through the live bird market, and researchers suggested better biosecurity to stop transmission [24–26]. Similarly, while finding the pathways of HPAI introduction into LBM in Indonesia, infected wild birds were identified as an essential risk factor [27]. The season has also been associated with an increased risk of exposure to AIV in LBMs. A significantly higher prevalence has been detected in the winter season compared to summer, according to previous studies conducted in Bangladesh [28,29]. Transmission of AIV in LBM is also associated with meteorological factors [30]. A study by Tang et al. [31] highlights the importance of relative humidity in AIV transmission, while a study by Ma et al. [32] suggests that temperature and wind speed are important factors in H5N1 outbreaks in China. Identifying specific risk factors and seasonal patterns in transmission can inform targeted interventions that maximize resource allocation in an otherwise resource-poor setting. Birds sold in Dhaka LBMs can come from different parts of a country; therefore, conducting AIV surveillance at these markets may be an effective strategy to monitor virus movement within a country. A contaminated environment can serve as a reservoir for the virus and remain infectious for several days [33]. This means that even healthy birds brought into the market can become infected, and people who work or visit the market can also be at risk of infection. So, we conducted this weekly longitudinal environmental surveillance in the LBM of Dhaka city to examine whether environmental sampling in LBMs could enhance the tracking of AIV virus subtype circulation in Dhaka and to identify the possible risk zones and climatic factors influencing the AIV transmission in LBM environments.

2. Methodology

2.1. Study design, location, and duration: a longitudinal study

AIV from commercial and backyard poultry farms aggregates across the country to a single location in LBM via poultry marketing. LBM environmental surveillance is a cost-effective and efficient method for detecting the presence of AIV in the country without visiting each individual poultry source. Hence, we deployed weekly longitudinal environmental surveillance in two LBMs in Dhaka city between January 2018 and March 2020. Tejgaon Railway market has a wholesale business (markets selling goods to retailers in larger quantities) type mostly, but sometimes some vendors also sell birds in small quantities. On the other hand, Kaptan Bazar has a mixed business type (markets selling goods to retailers in larger quantities and to final consumers in small quantities). In Dhaka city, we have selected two types of market as previous studies have shown that there might be differences in AIV circulation between wholesale and mixed markets [13,34].

2.2. Sample collection, preservation, and transportation

We divided each LBM into two risk zones: bird-holding and slaughtering zones. We hypothesized that slaughtering zones have a higher AIV subtype contamination risk than bird-holding zones. Considering this hypothesis, we collected separately one pooled fecal swab and one pooled offal swab sample for each LBM to boost the possibility of influenza virus detection. We collected 6 fecal samples using sterile polyester swabs with plastic shafts from the bird-holding zones at different stalls of each LBM and made them into a single fecal pool. In addition, we collected 6 offal swabs from slaughtering zones of different stalls of each LBM and made a single pooled offal swab sample from environmental surfaces at each LBM. The samples were collected in a 3.6 ml cryovial or 10 ml falcon tube containing 3 ml viral transport media (VTM) as previously described [35]. In the Lab, we stored the samples at −80 °C in the freezer until laboratory testing. The team wore gloves and N95 masks during sample collection at the LBMs.

2.3. Laboratory analysis

We extracted RNA using the magnetic bead-based RNA isolation technique in a KingFisher Flex 96-well robot with the MagMAXTM-96 AI/ND Viral RNA Isolation Kit (Applied BiosystemsTM, San Diego, CA) according to the manufacturer's instructions. We analyzed the pooled fecal and offal samples from each LBM separately for the Matrix (M) gene of the AIV virus. First, we used real-time reverse transcription PCR (rRT-PCR) with reference primers and probes to screen the swab samples for the presence of the M gene, as described previously [36]. The samples that tested positive for the M gene were then subtyped for the H5, H7, and H9 using hemagglutinin gene-specific primers and probes in the rRT-PCR assay [37]. The samples that tested positive for M-gene but negative for H5, H7, and H9 were classified as A/Untype.

2.4. Meteorological data

The markets enrolled in this study were classified as "semi-open," characterized by their exposure to direct sunlight and vulnerability to rainfall, resulting in a climate-dependent environment within the LBMs. So, to investigate the relationship between AIV contamination at the environmental surface on LBM and climatic factors, we collected meteorological data from the Bangladesh Meteorological Department (BMD) [38]. BMD records different meteorological data from 35 meteorological observatory stations across the country, including Dhaka city. We collected daily maximum temperature (°C), minimum temperature (°C), relative humidity (%), rainfall (mm), wind speed (knot), and cloud cover (hour/ day) from 2018 to 2020. The meteorological data that were provided by the Meteorological Department had missing records, and we used the interpolation imputations method to estimate these missing observations. The missing values were imputed using the mean of previous and subsequent values of the missing observations.

2.5. *Statistical analysis*

2.5.1. *Exploratory analysis*

We used exploratory analysis to determine the pattern of viral circulation across months, business types (wholesale and mixed), risk zones (bird holding zone and slaughtering zone), and sampling efforts (weekdays and weekends). The value of Cramer's V was then computed to assess if the season, business type, risk zone, sampling effort, and years may add multicollinearity to the multivariable model. The variables with Cramer's V values <0.50 were included in the multivariable model [39].

2.5.2. *Multivariable logistic regression using generalized estimating equation (GEE)*

Using GEE, we fitted a multivariable logistic regression model to determine the risk factors associated with the presence or absence of AIV, A/H5, and A/H9 viruses in the environment. Longitudinal panel data analysis often involves the examination of correlated observations within each subject [40]. In such cases, GEE can be an effective approach. The GEE method utilizes the correlation matrix to account for within-subject correlation when estimating regression coefficients. This approach is particularly useful when the data is clustered and the observations within each cluster are correlated. Consequently, we used GEE to estimate the logistic regression model's parameters in R version 4.2.0 within Rstudio version 2022.02.2 using the geepack package.

2.5.3. *Wavelet coherence analysis: one to one effect of meteorological factors on AIV contamination at LBMs*

In order to determine if two-time series oscillate simultaneously, wavelet coherence, one class of the wavelet transform method was employed to investigate the relationship of two-time series (meteorological variables, weekly AIV, and A/H5 cases) in time and frequency. The degree of wavelet coherence reveals how well one time series can forecast the other, and the phase connection between them shows the anticipated causal relationships [41].

2.5.4. *Multivariable modeling to identify meteorological risk factors*

We calculated Pearson's correlation coefficients between all-weather variables to identify potential multicollinearity problems (Supplementary Fig. S2). We found that humidity and rainfall are highly correlated ($p < 0.001$) (Supplementary Fig. S2). Also, humidity and minimum temperature are highly correlated ($p < 0.001$) (Supplementary Fig. S2). So, we used two sets of models to identify the effect of minimum temperature and humidity on AIV and A/H5 circulation. First, we used maximum temperature, wind, and humidity as independent variables to predict the weekly cases of AIV and A/H5 (Model 1). Then, we used minimum temperature and rainfall to predict the weekly cases of AIV and A/H5 (Model 2). We fitted models that assumed Poisson, negative binomial, and zero-inflated negative binomial for both models. The best-fitted model was selected based on the dispersion statistic and AIC (Supplementary Table S2 and Supplementary Table S3). We chose models with the lowest AIC and dispersion statistics close to 1. For the model with independent variables such as maximum temperature, wind, and humidity, negative binomial model, we have had dispersion statistics close to 1. So, we chose negative binomial regression for this model. For the model with minimum temperature and rainfall, Poisson regression had the lowest AIC along with dispersion statistics precisely as 1. So, for this model, we chose Poisson regression. Then we calculated and estimated the marginal mean (emmeans) [42] for the meteorological variables and used EMM plots to visualize the effect size of meteorological variables on the weekly cases of AIV and A/H5. We used R version 4.2.0 within Rstudio version 2022.02.2 and the package "ggeffects".

3. Results

3.1. *Weekly and monthly trends of AIV subtypes in LBM from 2018 to 2020*

The overall prevalence (across all time points and LBM) of AIV, A/H5, and A/H9 was 37.8% (95% CI: 33.3–42.2), 18.8% (95% CI: 15.3–22.34), and 15.4% (95% CI: 12.1–18.7) respectively. Figs. 1 and 2 illustrate the weekly and monthly circulation patterns of AIV subtypes in the environment from January 2018 to March 2020. Throughout the duration of the study, AIV was detected every month in the LBMs. December 2019 marked the peak of AIV circulation, as 86.67% of the sample was AIV-positive. A/H5 was detected during the whole duration of the study except for September 2018, November 2018, and April 2019–August 2019. From week 1 to week 9, we consistently detected A/H5 every week, and from week 1 to week 7, only A/H5 was detected in the environment of LBM. A/H9 was also detected throughout the study period. Every month from October 2018 to March 2020, A/H9 was detected in the LBM's surroundings. In 2018, only in August (week −31), co-circulation of A/H5 and A/H9 was detected. However, co-circulation of A/H5 and A/H9 was more prominent in 2019 and 2020. From October 2019 to March 2020, consistent co-circulation of A/H5 and A/H9 was detected. A/untyped was also reported in the LBMs during most of the study period. In September 2018, all samples were positive for the A/untyped virus. We also observed that from March 2018 to July 2018 and from May 2019 to September 2019, AIV was not detected for several weeks.

3.2. *Prevalence AIV subtypes graph monthly annual cycle: temporal and seasonal trends of AIV contaminations at LBM surfaces*

Fig. 3 shows the Temporal and Seasonal trends of AIV, A/H5, and A/H9 prevalence at LBM surfaces. The monthly prevalence of AIV ranged from 11.1%–71.8%. The highest prevalence of AIV was in December (71.8%; 95% CI: 56.1–87.7), and the lowest was in January (11.1%; 95% CI: 0.7–21.5). On the other hand, the monthly prevalence of A/H5 ranged from 3.8%–50%. The highest monthly prevalence of A/H5 was in February (50%; 95% CI: 35.7–64.2). May, June, August, and September had the lowest prevalence of A/H5 (3.8%). The monthly prevalence of A/H9 ranged from 27.8%–53.1%. The highest prevalence of A/H9 was in December (53.1%; 95% CI: 35.6–67), and the lowest was in May and September. For both AIV and A/H5, we can see that the prevalence in the summer (April–October) is lower than in the winter months (November–March). High seasonality is observed in the circulation of AIV and A/H5. However, in the circulation of A/H9, weak seasonality is seen across the annual cycle. Also, supplementary Fig. S1 shows that the prevalence of A/H5 is higher than A/H9 in the winter season (November–March).

3.3. *Prevalence of AIV subtypes by business type*

Prevalence of AIV was higher among the samples collected from mixed (40.7%; 95% CI: 27.6–53.7) business types than those with wholesale businesses (32.3%; 95% CI: 19.2–45.4) (Fig. 4). Similarly, the

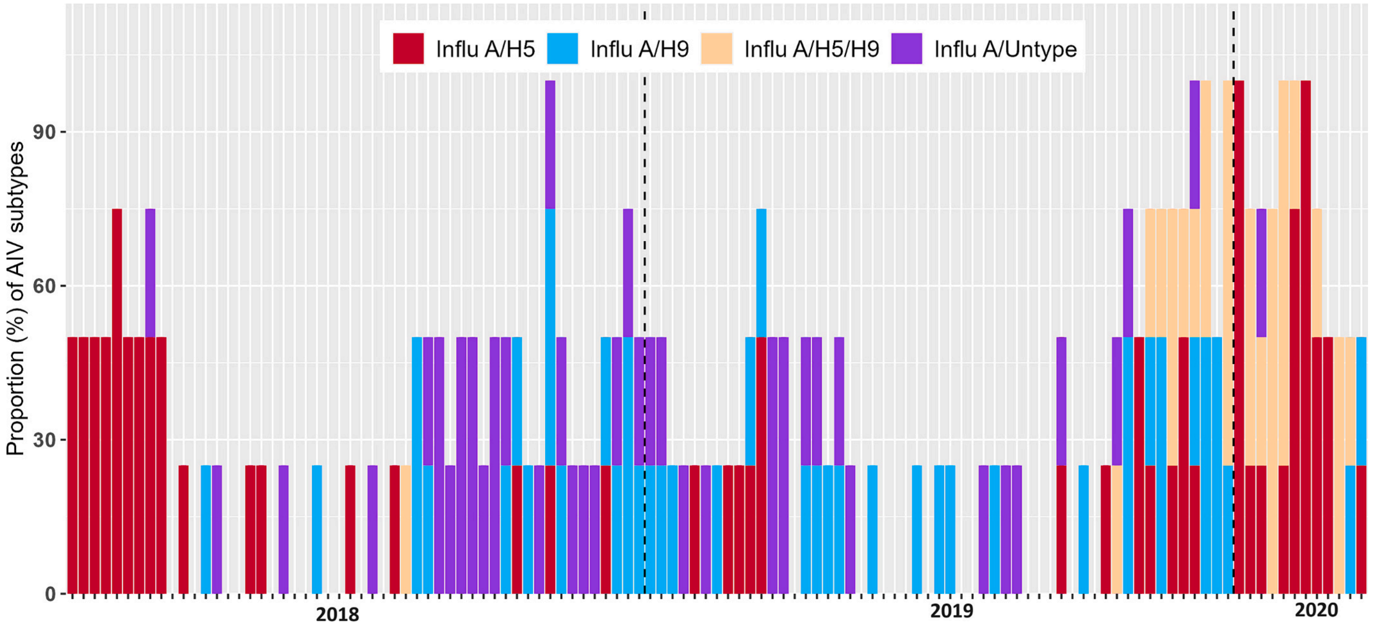

Fig. 1. Proportion of AIV subtypes weekly during 2018–2020. Each column comprises a week. The prevalence of each subtype each week is staked over each other where red bars indicate the proportion of A/H5 positive, blue bars A/H9 positive, yellow bars A/H5/H9 positive, and purple bars A/untype positive in each week. The dotted lines indicate the transition between years. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

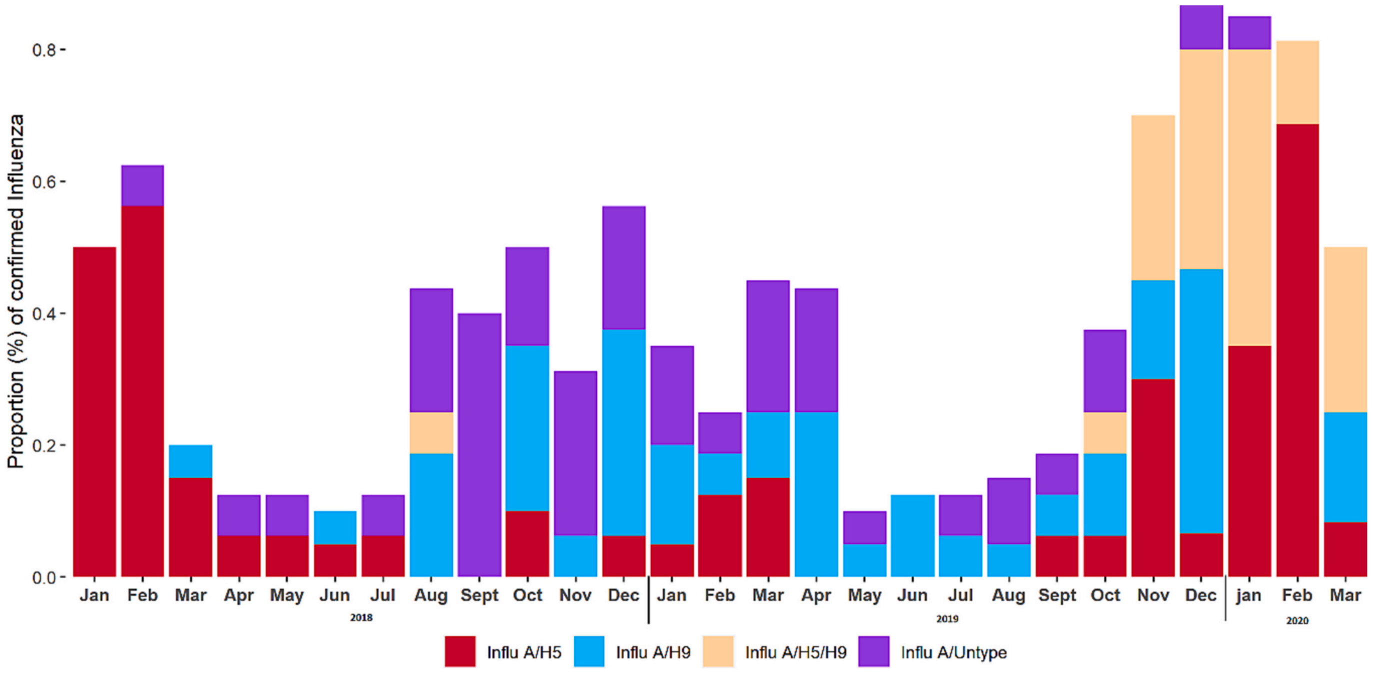

Fig. 2. Proportion of confirmed AIV subtypes each month during 2018–2020. The prevalence of each subtype each month is staked over each other where red bars indicate the proportion of A/H5 positive, blue bars A/H9 positive, yellow bars A/H5/H9 positive, and purple bars A/Untype positive in each month. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

prevalence of A/H5 was higher among the samples collected from vendors mixed (21.6%; 95% CI: 8.5–34.8) business type than those with wholesale businesses (13.3%; 95% CI: 0.2–26.4). However, in A/H9, the opposite pattern was observed. The prevalence of A/H9 was higher in mixed businesses (19.4%; 95% CI: 8.5–30.2) than in wholesale businesses (7.6%; 95% CI: 0–18.4).

3.4. Prevalence of AIV subtypes circulation by risk zoning at environmental surfaces at LBM

The prevalence of AIV and A/H5 was higher in the slaughtering zone than in the bird-holding zone. The prevalence of AIV in the slaughtering zone was 39.3% (95% CI: 33.0–45.6) (Fig. 5), and the bird-holding zone was 36.3% (95% CI: 31.2–42.5). Similarly, the prevalence of A/H5 in the slaughtering zone was 24.4% (95% CI: 18.9–29.9), and bird holding was

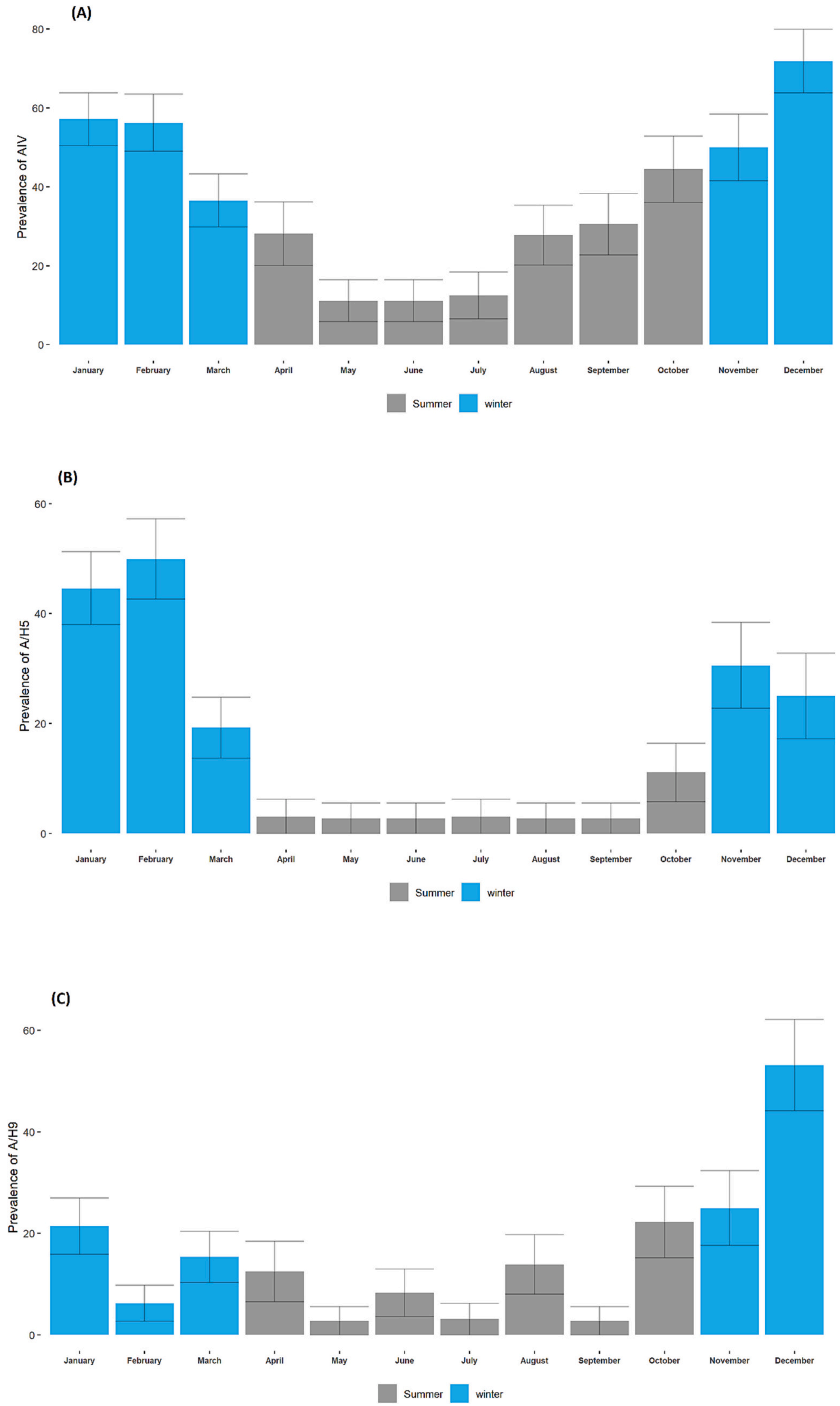

Fig. 3. (A) AIV prevalence in LBM across the annual cycle. (B) A/H5 prevalence in LBM the yearly cycle. (C) A/H9 prevalence in LBM across the annual cycle. Each plot's bars represent the prevalence for that particular month and include a 95% CI. The blue colored bars in each figure denote months that fall into the winter season, while the gray colored bars indicate months that fall into the summer season. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

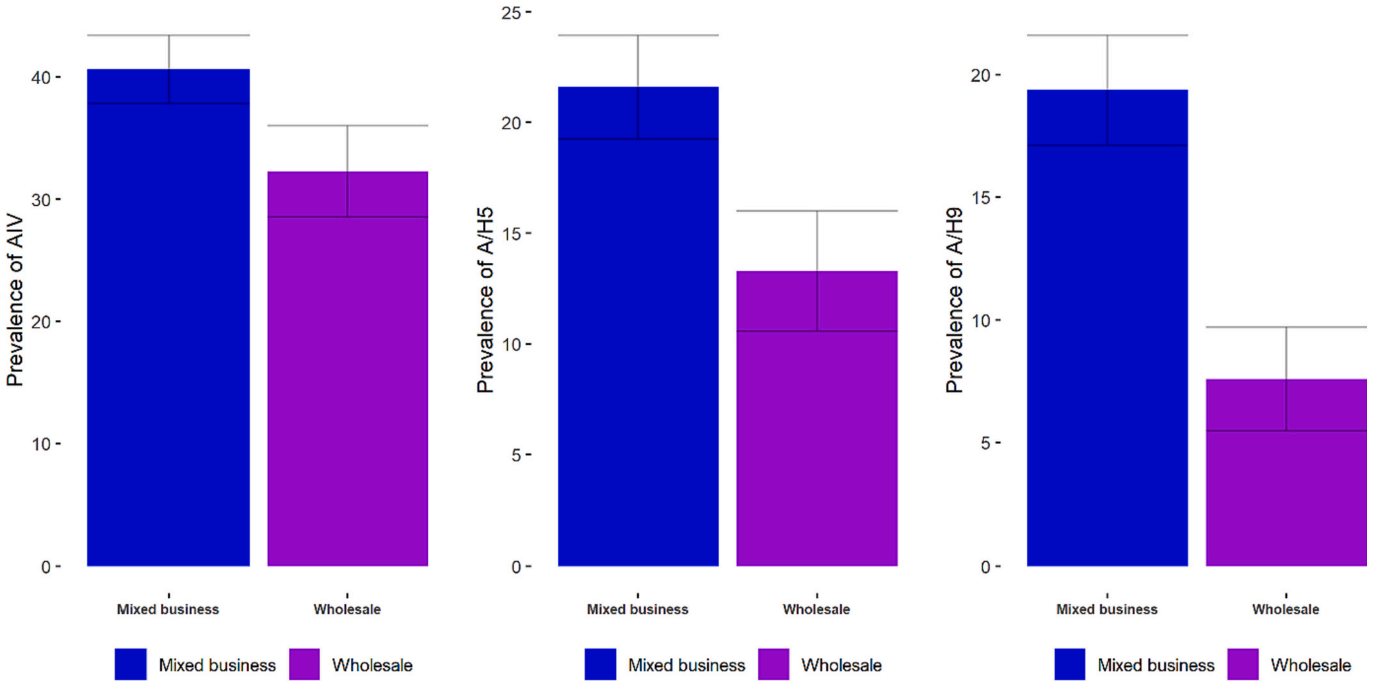

Fig. 4. Prevalence and 95% CI of AIV, A/H5, and A/H9 across the business type of market.

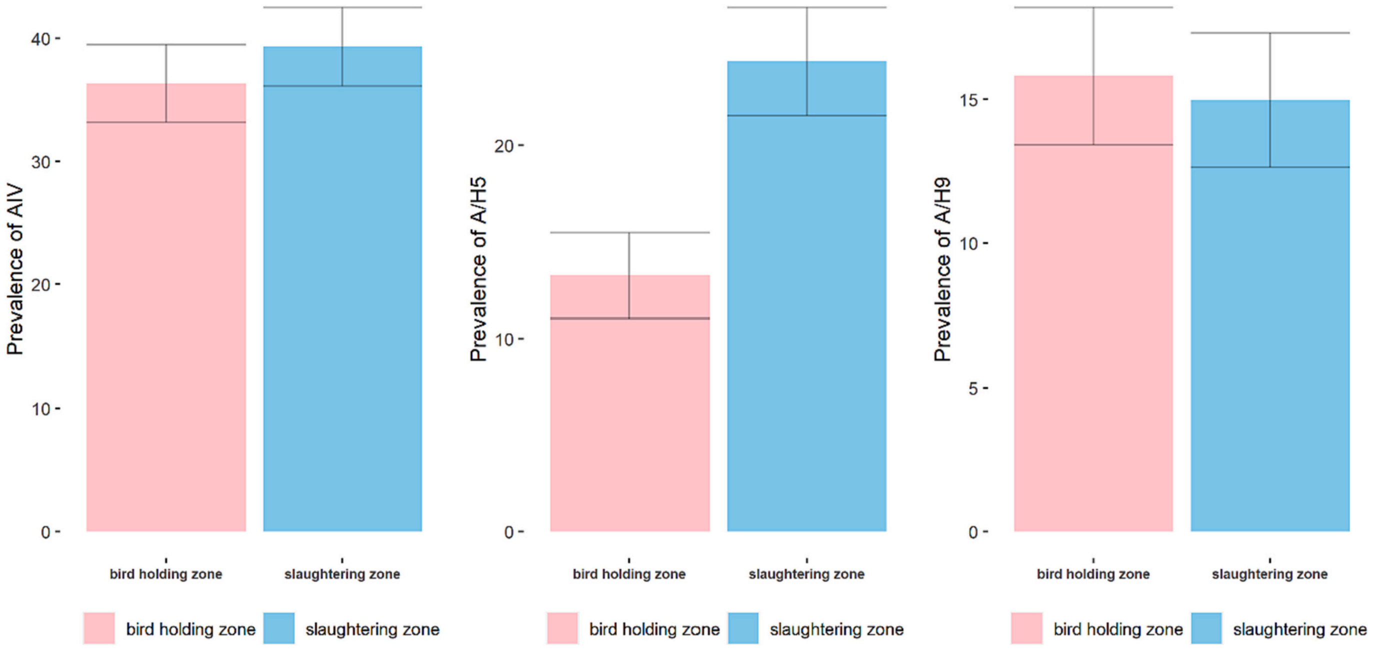

Fig. 5. Prevalence and 95% CI of AIV, A/H5, and A/H9 across the risk zones of the market.

13.2% (8.9–17.6). On the other hand, the prevalence of A/h9 was higher in the bird-holding zone (15.8%; 95% CI: 11.1–20.5) than in the slaughtering zone (15%; 95% CI:10.4–19.6).

3.5. *Prevalence of AIV and subtypes circulation by sampling efforts on weekdays and weekends*

The prevalence of AIV was higher on the weekend than on weekdays. The prevalence of AIV on the weekdays was 35.8% (95% CI: 27.2–44.5), and on the weekend, 38.5% (95% CI: 33.4–43.6) (Fig. 6). On the other hand, the proportion of A/H5 and A/H9 positive cases is higher on weekdays than on weekends. The prevalence of A/H5 on weekdays was 20.0% (95% CI: 12.8–27.18), and on weekends was 18.4% (14.3–22.5). On the other hand, the prevalence of A/h9 was 16.7% (95% CI: 10.0–23.4) on weekdays and 14.9% (95% CI: 11.2–18.7).

3.6. *GEE modeling to identify the associated risk factors*

According to our hypothesis, we took season, market type, risk zone, year, and sampling effort in our logistic regression. We calculated Cramer's V to check multicollinearity between the independent variables (Supplementary Fig. S2). Still, the values of Cramer's V for all the variables were <0.36. So, there would be no potential multicollinearity if we took all the variables in our model. We used GEE to estimate from

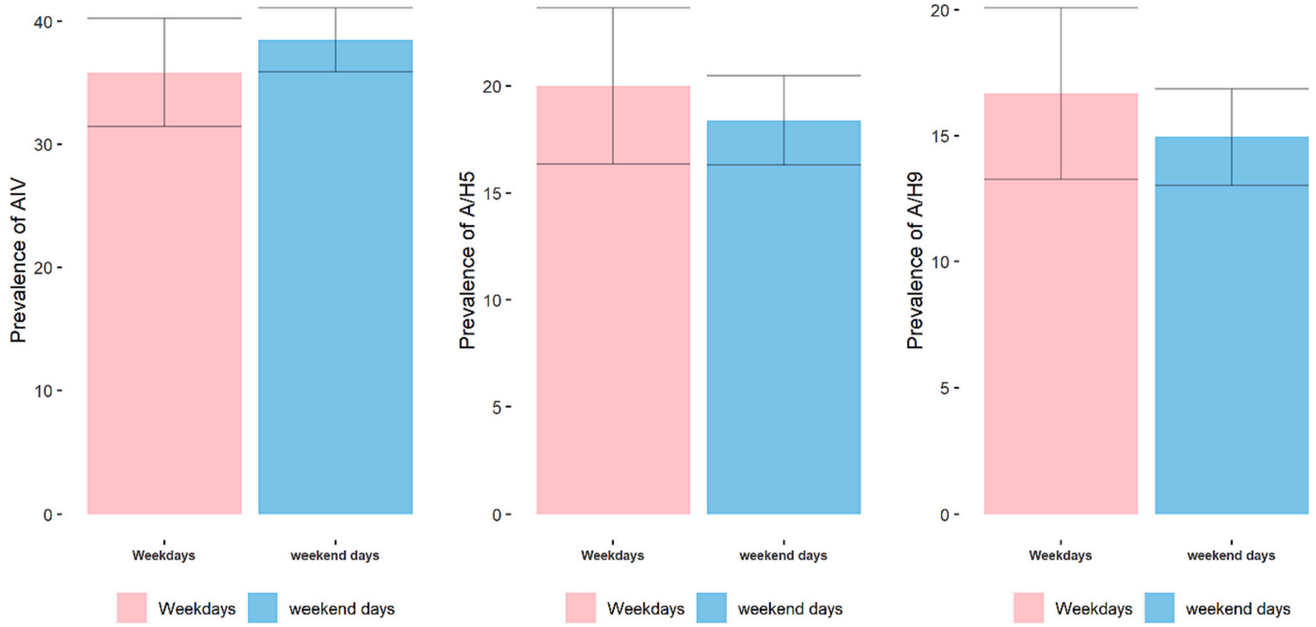

Fig. 6. Prevalence and 95% CI of AIV, A/H5, and A/H9 across the sampling efforts.

the multivariable logistic regression where LBM has been considered as cluster (Supplementary Table S1 and Fig. 7). AIV transmission is significantly influenced by monthly changes (Fig. 7 and Supplementary Table S1). The risk of AIV transmission is lower in the summer months, with the lowest transmission risk occurring between May and July. During these months, the odds of AIV transmission decrease by approximately 70–90% compared to the colder months, notably between November and February. In the months of March–April and August–September, the odds of AIV transmission also reduce by approximately 50–70% compared to the colder months. The second model developed to investigate the factors associated with the presence or absence of A/H5 viruses in the environment has revealed that both the risk zone and the month are significant factors (Fig. 7 and Supplementary Table S1). Specifically, the likelihood of detecting A/H5 in the environment is significantly lower in the months of April through September, with odds of 93–94% lower than in January.

Additionally, samples collected from the slaughtering zone were found to have almost three times the risk of testing positive for A/H5 compared to samples collected from the bird-holding zone. In the 3rd model, we can see that the presence or absence of A/H9 is significantly associated with monthly changes (Fig. 7 and Supplementary Table S1). In October and December, the risk of AIV detection was 4.36 times and 10 times higher, respectively, than in January.

3.7. *Temporal and seasonal dynamics of AIV contamination at LBM surfaces*

Here, we showed the time series decomposition of the weekly count of A/H5 data. We can see that there might be a seasonality among A/H5 shedding in LBM (Fig. 8). We used Wavelet coherence analysis and multivariable modeling to explore whether the seasonality in the shedding of A/H5 along with AIV depends on meteorological factors.

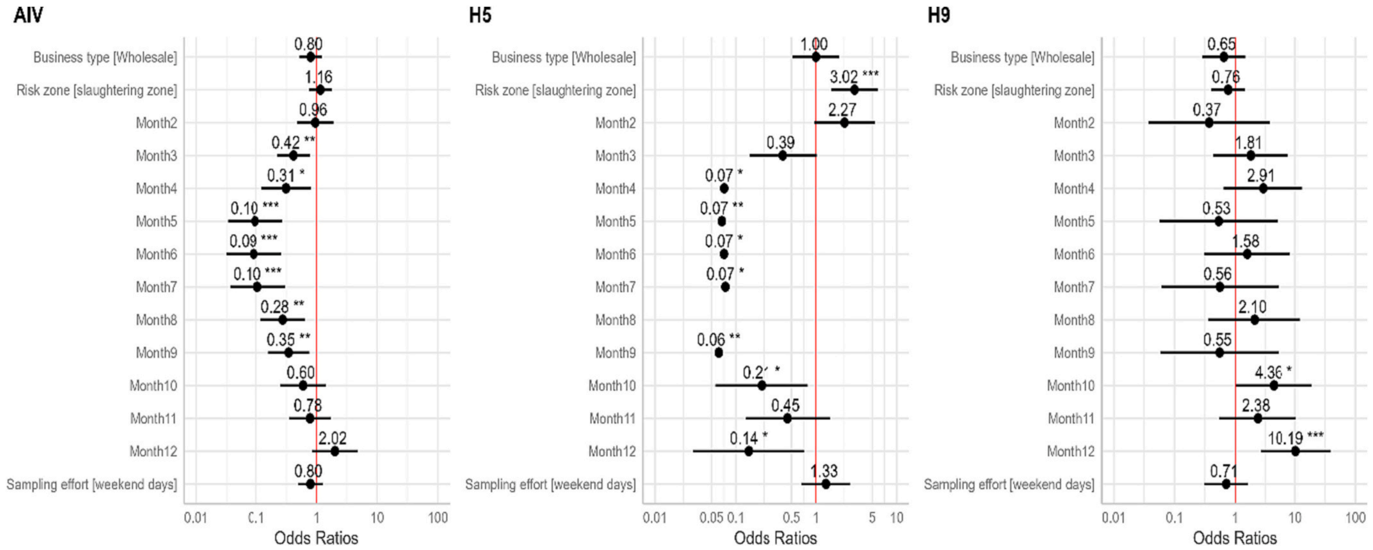

Fig. 7. Odds ratios of the presence of AIV, H5, and H9 as compared to reference category of each independent variable (Intercept, reference category not shown) with 95% confidence intervals and significance stars (*) from the GEE model is plotted. The "neutral" dotted line, i.e., the vertical intercept, indicates no effect (x-axis position 1 for Odds ratio).

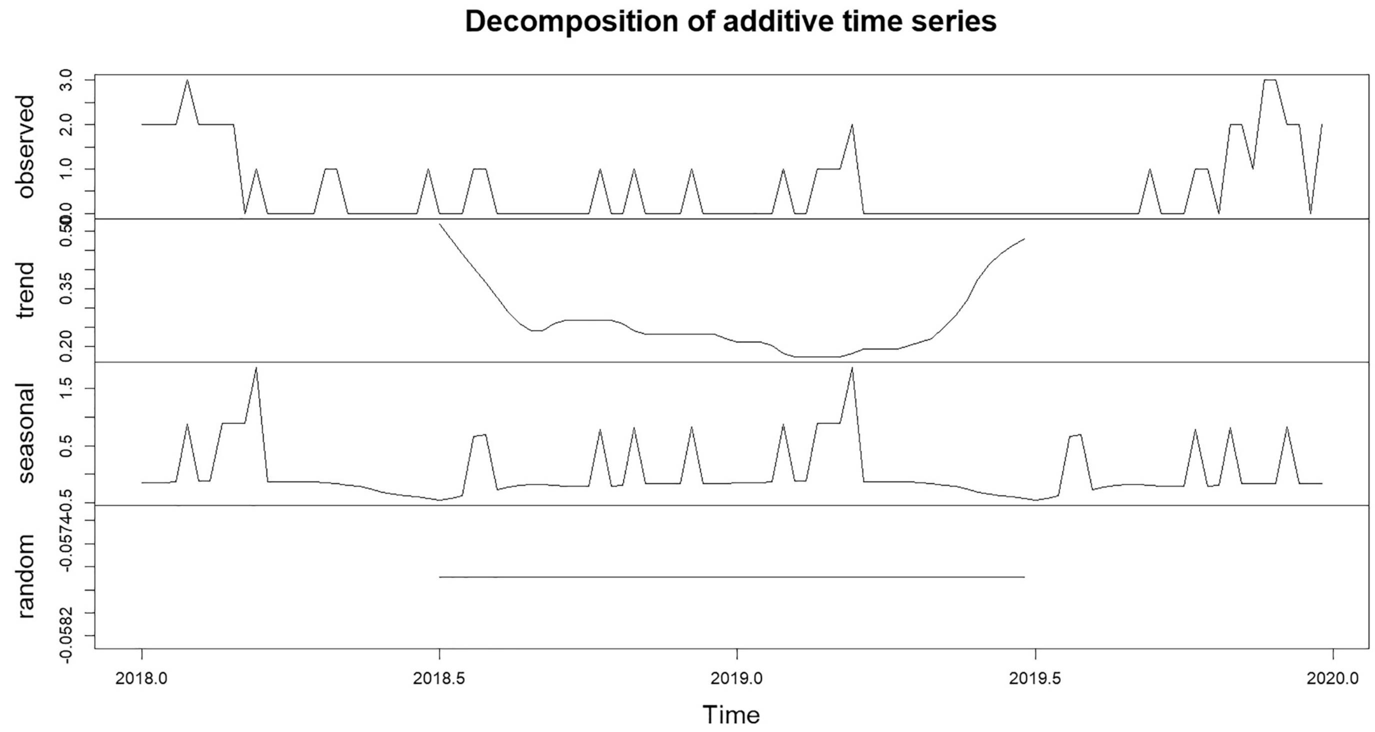

Fig. 8. Time series decomposition of weekly positive A/H5 count.

3.7.1. *Wavelet coherence analysis (correlation check climatic factors vs AIV circulation)*

We employed wavelet analysis to explore the association between the strong seasonality of weekly AIV and A/H5 cases and meteorological factors (Fig. 9, Fig. 10, Supplementary Fig. S3, and Supplementary Fig. S4). The scale on the right-hand side represents the matching of colors and correlation levels. The 5% significant level against red noise is shown as a thick black curve. Arrows indicate the phase difference between the two series. Arrows pointing to the right mean the variables are in phase [41]. Arrows pointing to the left mean that the variables are out of phase. The down arrows show that the climate factor is leading, and the up arrows mean that the influenza virus is leading. In-phase indicates that variables will have a cyclical effect on each other, and out-of-phase or anti-phase shows that variables will have an anti-cyclical impact on each other. We found that weekly maximum temperature, minimum temperature, rainfall, wind speed, and humidity presented a consistent association ($p < 0.05$) with the weekly AIV and A/H5 cases. We can see that these meteorological factors negatively correlate with the weekly cases of A/H5 (Fig. 9 and Fig. 10) and AIV (Fig. S3 and Fig. S4). We will proceed to further modeling to check if the factors combined affect the circulation of AIV and A/H5.

3.7.2. *Meteorological factors associated with AIV circulation in the environment*

We used the Pearson correlation test to remove multicollinearity between the exploratory meteorological variables. From supplementary Fig. S5, we found that weekly minimum temperature was highly associated with weekly maximum temperature and humidity. On the other hand, weekly humidity was also associated with weekly rainfall. So, we used two models to determine the effects of meteorological variables on the circulation of AIV and A/H5. The first model consisted of Maximum temperature, humidity, and wind speed. The second model consisted of minimum temperature and rainfall.

3.8. *Negative Binomial model for maximum temperature, relative humidity, and wind speed (Model 1)*

Table 1 shows that both maximum temperature and relative humidity are significantly associated with weekly cases of AIV and A/H5 ($p < 0.05$). To illustrate these effects in more detail, marginal means for these two explanatory variables are also plotted in Fig. 11 and Supplementary Fig. S6. We can see that maximum temperature ($\beta = 0.92$) and humidity ($\beta = 0.98$) both had a negative association with the weekly positive AIV cases (Fig. S6). A similar result is observed for A/H5 as the maximum temperature ($\beta = 0.86$) and humidity ($\beta = 0.96$) increase, and the weekly positive cases of A/H5 decrease (Fig. 11).

3.9. *Poisson model for climatic data with minimum temperature and rainfall (Model 2)*

From Table 2, we can see that minimum temperature is a significant factor in AIV and A/H5 circulation. To illustrate these effects in more detail, marginal means for these two explanatory variables are also plotted in Fig. 12 and Supplementary Fig. S7. We can see that minimum temperature has a negative association with both AIV ($\beta = 0.93$) (Fig. S7) and A/H5 ($\beta = 0.85$) (Fig. 12). As minimum temperature increases, weekly positive cases for AIV and A/H5 decrease.

4. Discussion

Our study, using intensive longitudinal surveillance in Dhaka, Bangladesh, represents the most comprehensive investigation of the recovery of AIV, H5, and H9 viruses in LBM work zones over 27 months. We addressed the limitations of earlier research conducted in LBMs in Bangladesh, particularly regarding the assessment of within-market environmental contamination. Our findings highlight the identification of potential risk zones, seasonal patterns, and meteorological factors that influence the transmission of AIV and H5 viruses, providing valuable insights for future efforts to mitigate the spread of these viruses in LBMs.

We identified the presence of AIV subtypes A/H5 and A/H9 in LBMs

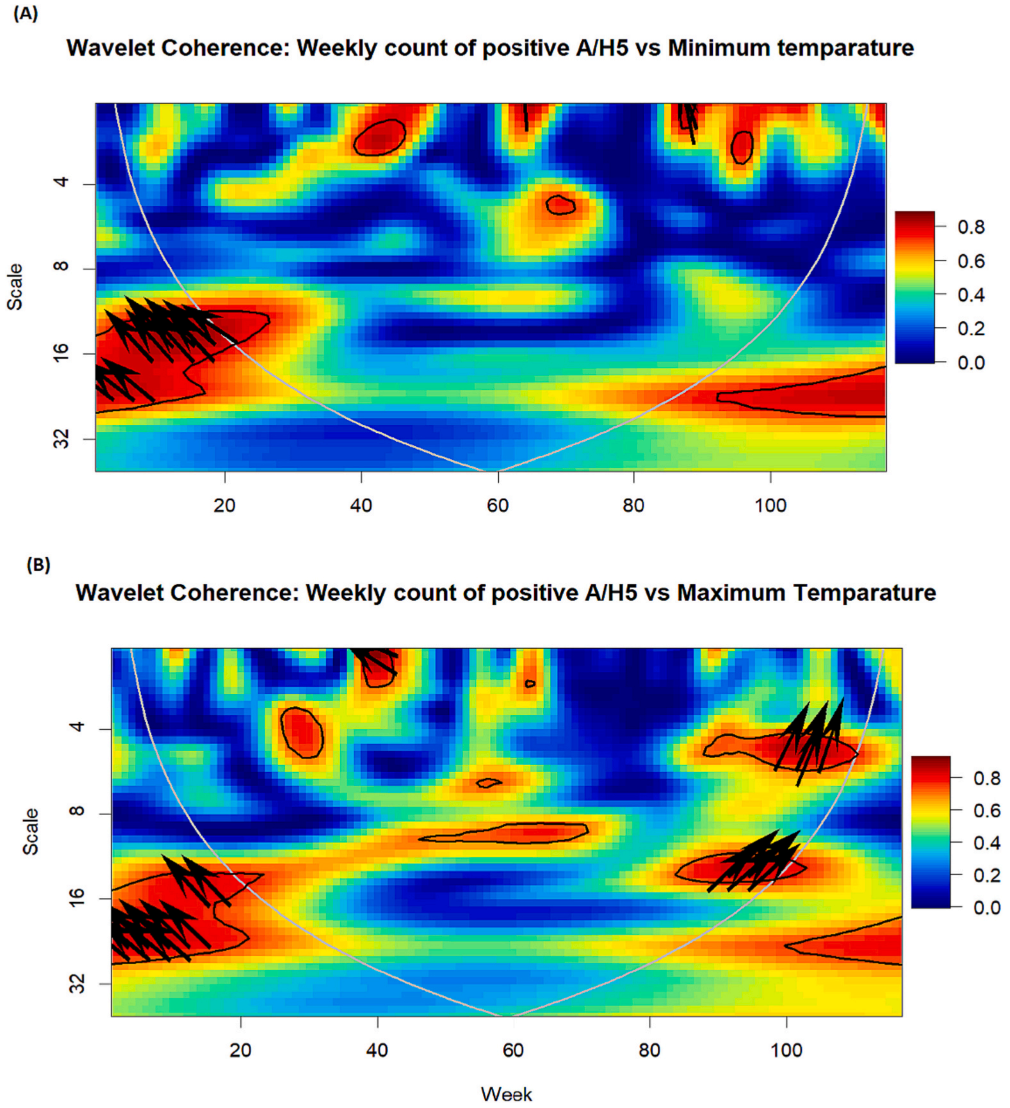

Fig. 9. (A) Wavelet coherence plot for A/H5 vs. Minimum temperature. (B) Wavelet Coherence plot for A/H5 vs. Maximum temperature. A colour spectrum indicates wavelet coherence. Red indicates high coherence, and blue indicates weak coherence as a function of the week of the study period (x-axis) and the oscillatory period (y-axis). Black lines indicate areas of coherence at a 5% significance level. Shaded areas represent regions where computed power spectra are less accurate due to boundary effects. Arrows pointing to the right mean that the variables are in phase. Arrows pointing to the left in our Figure indicate that the variables are out of phase. Downward arrows signify that the climate factor leads, while upward arrows indicate that the influenza virus leads in terms of their timing or influence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

throughout the year, consistent with findings from weekly surveillance in Cambodia and China [43,44]. While the overall prevalence of AIV in China was observed to be 10% higher than in our study. On the other hand, our findings revealed a higher prevalence of AIV in environmental samples from LBMs in Dhaka compared to earlier studies in Bangladesh [2]. The observed difference in prevalence may be attributed to our use of weekly surveillance, which is more robust and provides greater consistency in detecting AIV compared to the monthly surveillance utilized in the previous study. The prevalence of A/H5 in our study was also higher than in other AIV endemic countries like Egypt [45], Vietnam [46], Thailand [47] and Nigeria [48]. Despite the governments' repeated attempts to administrate H5N1 vaccine in commercial poultry farms in 2012, the detection and high prevalence of A/H5 in the LBMs suggests that an updated H5N1vaccination strategy may be required in endemic regions such as Bangladesh. It also indicates that the H5N1 virus is evolving silently [22,49] and requires rigorous monitoring to determine how to respond to this modification.

We detected LPAI virus A/H9 in the LBMs throughout the study period. Previous studies also noticed the circulation of A/H9 in the LBMs of Bangladesh [50,51] and other AIV endemic countries [52–54]. H9N2 viruses have been identified as donors or receivers of genes from different AIV subtypes, which may boost viral fitness in avian and mammalian hosts by overcoming host resistance [55], so we should be concerned with the continuous circulation of A/H9 in the LBMs of Bangladesh. The co-circulation of A/H5 and A/H9 was also identified throughout the study period. Particularly after the first half of 2019, consistent co-circulation of these two viruses was observed. It is a cause for concern since it may result in the emergence of new reassortant variations [56,57]. Detection of A/Untyped across the study period suggests that other HPAI and LPAI viruses may be prevalent in the LBMs of Bangladesh, which might result in economic losses due to poultry sickness. Surveillance in countries like Korea [58], Thailand [59], Nigeria [60], and Egypt [61] led to the detection of A/H6, A/H3, A/H1, A/H4, and other subtypes of AIV in the LBM. So, further intensive surveillance is needed to detect the subtypes of AIV circulating in the LBMs of Bangladesh. We identified a distinct yearly seasonal trend, with the peak of AIV and A/H5 occurring between November and March. Similar to our study, a previous study has revealed that Northern Temperate or

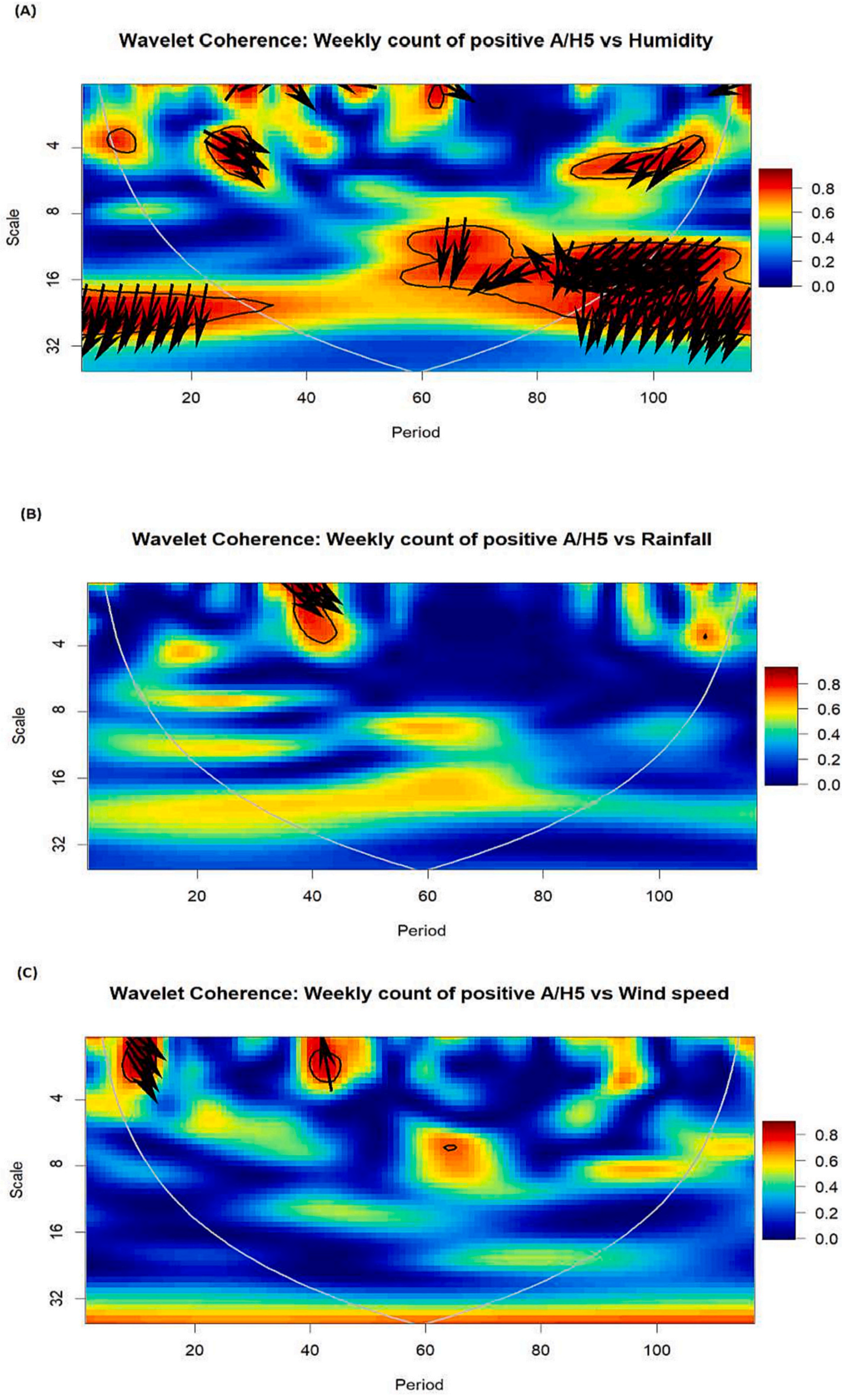

Fig. 10. (A) Wavelet coherence plot for A/H5 vs. Humidity. (B) Wavelet Coherence plot for A/H5 vs. Rainfall. (C) Wavelet Coherence plot for A/H5 vs Wind speed. A colour spectrum indicates wavelet coherence. Red indicates high coherence, and blue indicates weak coherence as a function of the week of the study period (x-axis) and the oscillatory period (y-axis). Black lines indicate areas of coherence at a 5% significance level. Shaded areas represent regions where computed power spectra are less accurate due to boundary effects. Arrows pointing to the right mean that the variables are in phase. Arrows pointing to the left in our Figure indicate that the variables are out of phase. Downward arrows signify that the climate factor leads, while upward arrows indicate that the influenza virus leads in terms of their timing or influence. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Subtropical countries such as Bangladesh, China, Egypt, and Turkey and tropical countries such as Cambodia, Indonesia, and Vietnam saw the highest peaks of H5N1 from January to March [62]. However, the timing of the peak outbreak may vary in certain regions, such as Thailand, where it occurs in October [63].

The GEE model also revealed that the presence of AIV and A/H5 in environmental samples was significantly higher in colder months than in summer. Similar to our findings, AIV-endemic Egypt had higher odds of

Table 1

Association of Maximum temperature, Humidity, and Wind with AIV and A/H5 occurrence at LBM environment through Negative binomial model.

	AIV			H5		
Predictors	Incidence Rate Ratios	CI	p	Incidence Rate Ratios	CI	p
Maximum Temperature	0.92	0.90–0.96	**<0.001**	0.86	0.81–0.90	**<0.001**
Relative humidity	0.98	0.97–1.00	**0.011**	0.96	0.94–0.98	**<0.001**
Wind Speed	1.02	0.90–1.16	0.756	1.19	0.96–1.46	0.106

positive H5N1 virus infections in LBMs in the winter [64]. According to Park and Glass [65], the risk of avian and human influenza seems to be greater in East and Southeast Asia throughout the winter. Similarly, a previous study in Japan [66] also found that winter was associated with a higher prevalence of H5N1 infections and comparable findings have been reported in wild and domestic birds in Korea [67]. We discovered that both the holding area for birds and the slaughtering area had a substantial prevalence of AIV and A/H5 viruses. This suggests both zones are risky, and the market's surface is highly contaminated. It is recommended that the LBM committee take measures to disinfect the LBM surface. According to our model, there is a significantly higher risk of A/H5 in the samples from the slaughtering zone. Slaughtering creates droplets that may contain viral particles and expose internal organs to potentially high viral loads, and this contamination is to be anticipated. Secretions, internal tissues, and organs with potentially high virus loads may also widely splash out and spread throughout a poultry stall's narrow and poorly ventilated interior. A higher chance of AIV in slaughtering areas was also found in LBMs in China and Indonesia [68,69].

On the other hand, in Cambodia and Egypt, where the practice of on-site slaughtering of birds in LBMs is common, efforts have been made to promote safe slaughtering practices and to encourage the use of centralized slaughterhouses instead of LBMs to reduce the transmission of H5N1 [70,71]. To reduce the infection risk at LBM, our findings suggest that the practice of slaughtering live poultry in LBMs be avoided and a centralized poultry slaughtering plant be explored as an alternative. The cultural preference of purchasing freshly slaughtered poultry makes it an ideal environment for A/H5 to be introduced, transmitted among avian species, and even infect humans. Despite this, the availability of dressed poultry in urban areas is growing nowadays. It is plausible that the live poultry market may one day be converted into a dressed poultry market if the government were to raise public knowledge of the dangers of transmitting infectious diseases when slaughtering live birds. According to our findings, there was no significant change in the circulation of AIV or A/H5 on the surface of LBMs during the weekdays and the weekends. We hypothesized that since people tend to shop more on weekends than weekdays, there is an increased chance of animal and human interaction [72]. However, we could not detect any variation in the circulation of viruses between the weekdays and the weekends. This might be because the surface of the LBM is not sufficiently cleansed, and the virus can survive on the surface of LBMs infected over the weekend [73]. Our suggestion is to have one day during which the LBM is closed so that the surface of the LBM may be disinfected and contamination reduced. We investigated the climatic factors that can influence the survivability and persistence of AIV and A/H5 in the environment and contribute to their spread. We found that the temperature of the day and relative humidity were associated with the incidence rate of AIV and A/H5. Previous studies have shown an association between environmental parameters such as temperature, humidity, and influenza virus transmission [74] [75,76]. We found from the multivariable model that the incidence rate of AIV and A/H5 increases as a day's temperature decreases. We also saw from the wavelet coherence plot that minimum temperature, maximum temperature, and AIV and A/H5 are negatively associated. A previous study reported that AIV replication rises at lower temperatures [77], and colder temperatures may allow for more prolonged viral survival in secretions and feces of infected poultry [62,77]. Paek, Lee [78] also showed that increasing temperature decreases the chance of A/H5 virus survival. And Jaakkola, Saukkoriipi [79] showed that in Finland, a 1 °C drop in temperature was associated with an 11% increase in the predicted risk of influenza. We also showed that the incidence rate of both AIV and A/H5 increases as the relative humidity decreases. A previous study in the continental United States [80] have shown that seasonal changes in influenza transmission are caused by low absolute humidity. Another study in Japan showed an increased influenza incidence in high humidity [81]. Temperatures and humidity in Bangladesh are lower between November and March than during the other months. The climatic factors might be the reason for seasonality in the circulation of AIV and A/H5 in the environment of LBM. Wind and rain speed did not affect the circulation of AIV or A/H5 on the surface of LBM in our study. On the other hand, Chen, Zhang [82] showed that the H5N1 epidemic is negatively impacted by wind speed. The H5N1 virus epidemic is decreased with high wind speed as it improves ventilation in farms, marketplaces, and other live poultry farms in China. On the other hand, in Nigeria, low rainfall is associated with a higher prevalence of AIV [83]. The variation may be caused by Bangladesh's climate, which differs from the climates of these other nations. Since the ambient contamination of LBMs in Dhaka might not reflect the regional peculiarities of LBMs in different cities in Bangladesh, care should be given to evaluation.

5. Conclusions

Our Environmental surveillance demonstrates a significant prevalence of AIV subtypes with H5 and H9 circulating in LBM in Bangladesh throughout the year. We also identified that HPAI H5 was circulating in the environment of the LBM in the winter months significantly, which poses a threat to poultry and public health. Slaughtering zones in LBMs significantly increase the likelihood of avian influenza virus contamination compared to bird-holding zones. This study highlights significant relationships between the weekly circulation of AIV in LBMs and the meteorological variables temperature and relative humidity. Maintaining weekly market rest days and slaughtering zone custom interventions in LBMs should be considered in reducing AIV contamination at the market level. We suggest environmental surveillance as an early warning tool for understanding seasonal and temporal trends and detecting the emergence of a novel AIV strain in Bangladesh. The high prevalence of AIV in LBMs suggests that current control strategies may be ineffective. Traditional outbreak-based surveillance may not be sufficient for monitoring and controlling HPAI, as it may not detect infections until they have already spread. Therefore, it is important to implement one health avian influenza surveillance in the poultry-human interface and improve biosecurity measures in poultry farms and LBMs to reduce the spillover of HPAI H5 in humans in Bangladesh.

Ethical approval

The study protocol was approved by the Chattogram Veterinary and Animal Sciences University- Ethics Committee (protocol: CVASU/Dir (R&E) EC/2015/1011(4)) to carry out the project activities.

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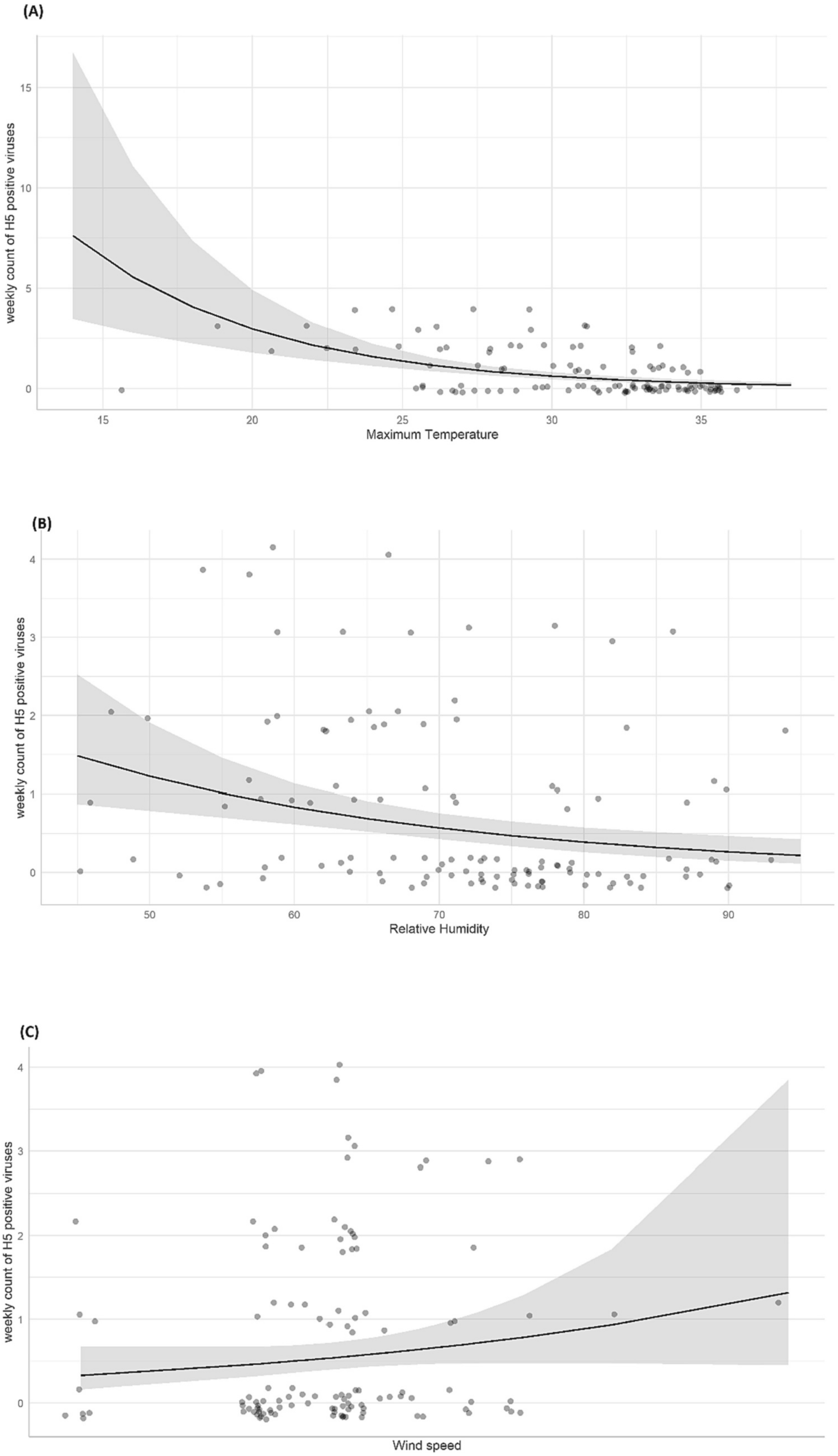

Fig. 11. In panel A-C, relationships of Maximum temperature, relative humidity, and wind speed with A/H5 circulation are depicted, using the partial residuals of the response variables and depicting the marginal effect response curve for each relationship.

Table 2
Association of minimum temperature and rainfall with AIV and A/H5 occurrence at LBM environment through Poisson model.

	AIV			H5		
Predictors	Incidence Rate Ratios	CI	p	Incidence Rate Ratios	CI	p
Minimum Temperature	0.93	0.90–0.95	**<0.001**	0.85	0.81–0.89	**<0.001**
Rainfall	0.99	0.97–1.00	0.132	0.98	0.94–1.01	0.346

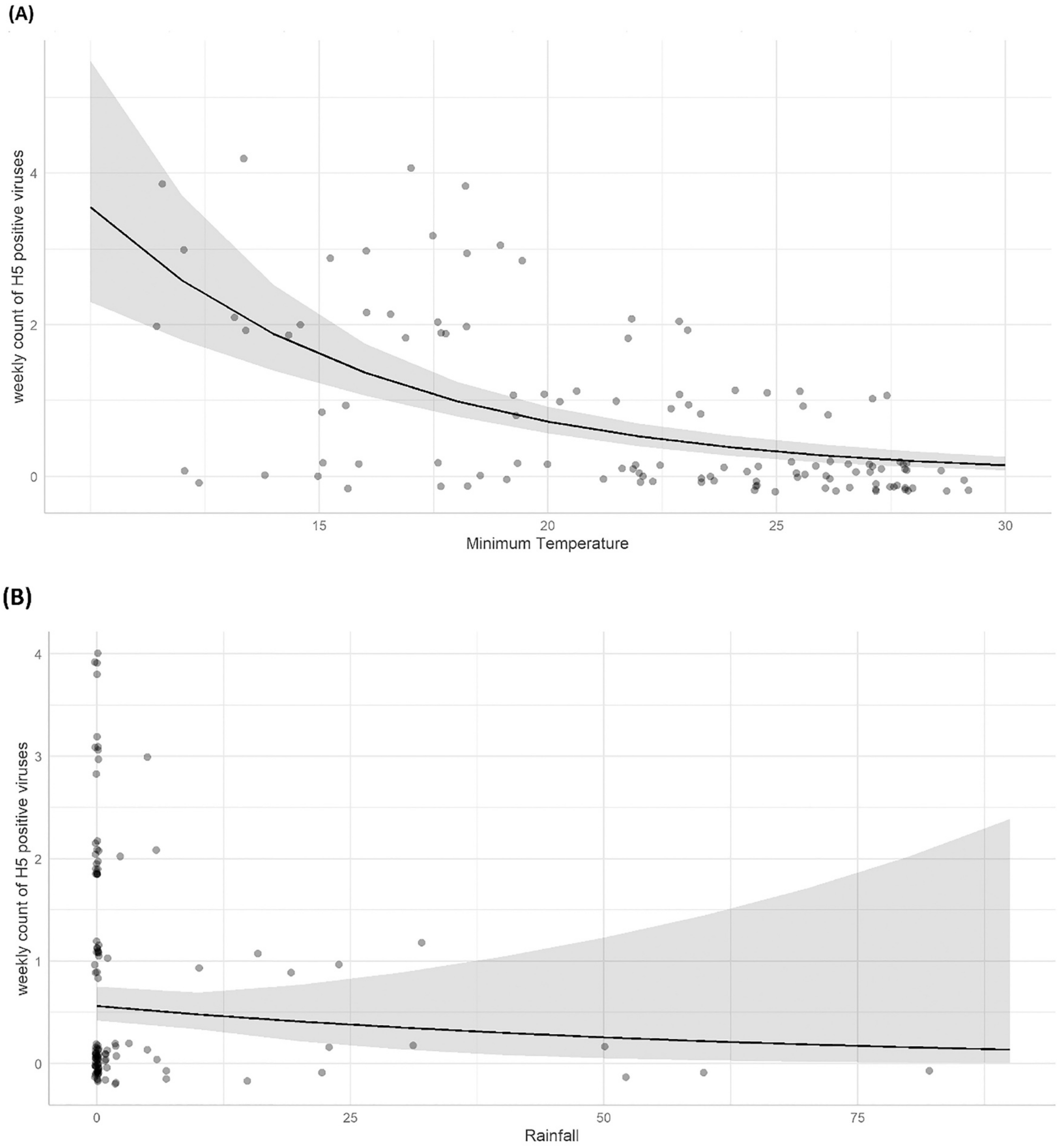

Fig. 12. In panel A-B, relationships of Minimum temperature and Rainfall with A/H5 circulation are depicted, using the partial residuals of the response variables and depicting the marginal effect response curve for each connection.

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CRediT authorship contribution statement

Ariful Islam: Conceptualization, Data curation, Formal analysis, Investigation, Methodology, Software, Writing – original draft. **Emama Amin:** Data curation, Formal analysis, Visualization, Writing – review & editing. **Sarah Munro:** Validation, Visualization, Writing – review & editing. **Mohammad Enayet Hossain:** Methodology, Investigation, Writing – review & editing. **Shariful Islam:** Methodology, Investigation, Writing – review & editing. **Mohammad Mahmudul Hassan:** Data curation, Methodology, Writing – review & editing. **Abdullah Al Mamun:** Data curation, Methodology, Investigation, Writing – review & editing. **Mohammed Abdus Samad:** Investigation, Methodology, Resources, Writing – review & editing. **Tahmina Shirin:** Resources, Supervision, Project administration, Funding acquisition, Writing – review & editing. **Mohammed Ziaur Rahman:** Resources, Investigation, Project administration, Funding acquisition, Writing – review & editing. **Jonathan H. Epstein:** Conceptualization, Methodology, Resources, Funding acquisition, Supervision, Project administration, Writing – review & editing.

Declaration of Competing Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Data availability

The original contributions presented in the study are included in the article/supplementary material; further inquiries can be directed to the corresponding author.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.onehlt.2023.100644.

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