ORIGINAL ARTICLE

Patterns of Avian Influenza A (H5) and A (H9) virus infection in backyard, commercial broiler and layer chicken farms in Bangladesh

Short running title

Patterns of endemic avian influenza virus infection

Suman Das Gupta[1], Md. Ahasanul Hoque[2], Guillaume Fournié[3], Joerg Henning[1]

[1]School of Veterinary Science, University of Queensland, Australia [2]Chattogram (previously Chittagong) Veterinary and Animal Sciences University, Bangladesh [3]Department of Pathobiology and Population Sciences, Royal Veterinary College, University of London, United Kingdom

Summary

In order to control Highly Pathogenic Avian Influenza (HPAI) H5N1 and Low Pathogenic Avian Influenza (LPAI) H9N2 virus spread in endemically infected countries, a detailed understanding of infection patterns is required. We conducted cross-sectional studies in Bangladesh in 2016 and 2017, on 144 backyard, 106 broiler and 113 layer chicken farms. Although all sampled birds were negative for H5 virus by RT-PCR, H5 antibodies were detected in unvaccinated birds on all three farming systems. Higher H5 antibody prevalence was observed in ducks raised on backyard farms, 14.2% (95% CI: 10.0-19.8%), compared to in-contact backyard chickens, 4.2% (95% CI: 2.8-6.1%). The H5 antibody prevalence was lower in broiler chickens, 1.5% (95% CI: 0.9-2.5%), compared to layer chickens, 7.8% (95% CI: 6.1-9.8%). H9 viruses were detected by RT-PCR in 0.5% (95% CI: 0.2-1.3%) and 0.6% (95% CI: 0.3-1.5%) of broilers and layers respectively, and in 0.2% (95% CI: 0.0-1.2%) of backyard chickens. Backyard chickens and ducks showed similar H9 antibody prevalence, 16.0% (95% CI: 13.2-19.2%) and 15.7% (95% CI: 11.3-21.4%), which was higher compared to layers, 5.8% (95% CI: 4.3-7.6%), and broilers, 1.5% (95% CI: 0.9-2.5%). Over the course of a production cycle, H5 and H9 antibody prevalence increased with the age of backyard and layer chickens. Usually, multiple ducks within a flock were H5 antibody positive, in contrast to backyard chickens, broilers and layers where only individual birds within flocks developed H5 antibodies. Our findings highlight low virus circulation in healthy chickens of all production systems in Bangladesh, which is in contrast to high virus circulation reported from live bird markets. Data generated in this project can be used to adopt risk-based surveillance approaches in different chicken production systems in Bangladesh and to inform mathematical models exploring HPAI infection dynamics in poultry from the source of production.

KEYWORDS

HPAI, H5, H9, infection pattern, antibody prevalence, virus prevalence

1 | INTRODUCTION

Highly Pathogenic Avian Influenza (HPAI) H5N1 virus is considered to be endemic in Bangladesh, China, Egypt, India, Indonesia and Vietnam (CDC, 2019; FAO, 2011), causing sporadic cases in humans, generally associated with exposure to infected poultry or contaminated environments (Fournié, Høg, Barnett, Pfeiffer, & Mangtani, 2017). In addition, it is feared that the ongoing co-circulation of Low Pathogenic Avian Influenza (LPAI) virus subtype H9N2 in H5N1-endemic areas might promote the emergence of reassortants able to spread effectively among humans (Marinova-Petkova et al., 2016; Parvin et al., 2019; Parvin et al., 2018; Thuy et al., 2016). In fact, out of the 861 H5N1 (as of January 2020) and 59 H9N2 (as of June 2019) laboratory-confirmed human cases in the world, 86.8% (747) H5N1 (Bangladesh: 8, China: 53, Egypt: 359, India: 0, Indonesia: 200, Vietnam:127) and 86.4% (51) H9N2 (Bangladesh: 3, China: 44, Egypt: 4, India:0, Indonesia: 0, Vietnam: 0) cases were reported in endemically infected countries (Peacock, James, Sealy, & Iqbal, 2019; WHO, 2020).

H5N1 infection had also an severe impact on poultry populations in endemically infected countries, for example, resulting in Bangladesh in the death and culling of more than 2.7 million poultry between 2007 and 2019 (DLS, 2019). However, the average number of reported poultry outbreaks is declining in Bangladesh, i.e. from 83 and 10 outbreaks in commercial and backyard poultry in 2007-12, to 2 and 0 respectively, in 2013-19 (DLS, 2019). Underreporting might be one reason for this decline as compensation policies were discontinued (Chattopadhyay et al., 2018) or because farmers might have accepted the ubiquity of HPAI outbreak occurrence similar to the endemicity of Newcastle Disease in many developing countries (Spradbrow, 1996).

Investigations of HPAI outbreaks have generated insights in possible risk factors associated with sudden deaths of birds (Biswas et al., 2009; Loth, Gilbert, Osmani, Kalam, & Xiao, 2010; Osmani et al., 2014), but they don’t provide information about the circulation of avian influenza virus in farmed poultry populations in endemically infected countries. Additionally, studies aiming to assess the level of viral circulation in poultry are generally conducted in live bird markets (LBM) (ElMasry et al., 2017; Kim et al., 2018; Negovetich et al., 2011; Thuy et al., 2016), and rarely on poultry farms. This can partly be explained by the ease of sampling at, as birds raised under different production systems are brought

together in a single market location. However, prevalence of infection estimated from marketed poultry populations cannot be extrapolated to farmed populations. Furthermore, although research has focussed on range of farmed poultry such as backyard poultry (Henning et al., 2011 Khatun et al., 2013; Nooruddin, Hossain, Mohamma, & Rahman, 2006), nomadic or stationary ducks (Haider et al., 2015; Henning et al., 2010; Sarkar et al., 2017) and commercial chickens (Ansari et al., 2016; Haque, Kabir, Ali, Rahman, & Islam, 2015), a comparison of H5 and H9 infection status between poultry production systems has not been conducted.

In Bangladesh, about 80-90% of rural households (HHs) rear small flocks of poultry in their backyard. These backyard chickens are referred to as Deshi ('indigenous' in Bangla) and they are usually reared under scavenging or free ranging conditions (Barua & Yoshimura, 1997; FAO, 2008). Many backyard chicken farmers also rear ducks, and sometimes pigeons and geese (Alam, Ali, Das, & Rahman, 2014; FAO, 2008). In contrast, commercial broiler and layer farmers raise exotic strains or cross-breeds of chickens (e.g. Cobb 500 strain, Hisex brown strain, Sonali cross-breed) under confinement, with provision of commercial poultry feed (FAO, 2008; Huque, Saleque, & Khatun, 2011).

In order to control and prevent the spread of H5N1 and H9N2 viruses in chickens, a detailed understanding of infection patterns at bird and flock-level is required. It is hypothesized that different poultry species as well as different poultry husbandry systems might play different roles in the transmission and maintenance of avian influenza viruses (Alexander, 2000; Zhang et al., 2014). The study presented here quantifies the extent of H5 and H9 virus circulation on backyard chicken farms, and on commercial broiler and layer chicken farms in two representative districts of Bangladesh. We estimated 1) bird and flock-level prevalence of current and past H5 and H9 infection, 2) the magnitude of spread of the infection within flocks, 3) variations in antibody prevalence with age and 4) the spatial distribution of H5 and H9 infection in backyard flocks.

2 | MATERIALS AND METHODS

2.1 | Targeted population

Chittagong is the second largest city in Bangladesh representing 19.7% of the country's urban population and generating 30% of Bangladesh's national GDP (BBS 2011c; Hassan & Nazem, 2016). Chittagong and Cox's Bazaar districts are the two main suppliers' of chickens to the Chittagong City Live Bird Markets (CCLBM) (Moyen, 2019; Moyen et al., 2018). A total of 1796 (1507 broiler and 289 layer) and 627 (397 broiler and 230 layer) farmers rear commercial broiler and layer chickens in the Chittagong and Cox's bazaar districts, respectively (DLS, 2014). In addition, an estimated 0.7 and 0.3 million households rearing backyard poultry in the Chittagong and Cox's bazaar districts (BBS, 2014; BBS, 2015) providing an important income for livelihood of the rural population (FAO, 2008). Due to the national importance of poultry production and trade in these two districts, farms in the Chittagong and Cox's Bazaar districts were considered as the target population for this study.

2.2 | Study design

Two cross-sectional studies were conducted in the Chittagong and Cox's Bazaar districts of Bangladesh. Backyard chicken farms were visited between February and April 2016, and commercial broiler and layer chicken farms between February and April 2017.

2.2.1 | Sample size

For the sample size calculations, H5 bird- and flock-level antibody prevalence were assumed to differ between poultry species and production systems. For each poultry species (i.e. ducks and chickens) and each production system (i.e. backyard, commercial broiler and layer), a two stage sampling approach was used to estimate 1) the number of farms, and 2) the number of birds per farm to be sampled (Humphry, Cameron, & Gunn, 2004). Input parameters for sample size calculations and estimated sample sizes are listed in Supplementary Table 1. The assumed design prevalence, i.e. the expected bird and flock-level H5 antibody prevalence for backyard and commercial birds, were based on Henning et al. (2011) and Hassan (2017), respectively.

2.2.2 | Sampling approach

2.2.2.1 | Selection of administrative areas

The selection of sub-districts (upazillas) for the sampling of backyard farms in the Chittagong district was based on features identified to influence avian influenza viral transmission (Ahmed et al., 2012), which included: 1) Density of backyard poultry farms, 2) Density of backyard chickens, 3) Location of the sub-districts within the district, 4) Environmental characteristics of the sub-district, and 5) Distance of the sub-district to Chittagong City, where most live bird markets are located (Supplementary Table 2). The density of backyard poultry farms per square kilometre was calculated from census data: it was based on the number of rural households (BBS, 2014, 2015), assuming that 80% of households raise backyard poultry (FAO, 2008). The density of backyard chickens was also based on census data (BBS, 2011a, 2011b). Quartiles of backyard farm density and backyard chicken density were computed and each sub-district was assigned to one of those quartiles. To cover most of a district's geographical area, the Chittagong district was divided into regions (south, north, east, west, middle), and sub-districts were identified from each of these regions. Furthermore, we classified subdistricts according to the presence of water reservoirs (rivers/canals/access to the sea), woodlands (forest/hill), and the sub-district's centroid distance to Chittagong City. A ranking matrix was then developed for all sub-districts in the Chittagong district, and eight sub-districts were selected representing combinations of all five selection criteria. The two sub-districts in the Cox's Bazaar district, which were the main suppliers of poultry for CCLBMs, were also selected (Moyen, 2019). The same sub-districts selected for the backyard farm sampling were also used for the sampling of commercial layer and broiler farms.

2.2.2.2 | Selection of villages and backyard chicken farms

Quartiles for the number of households (or backyard farms) per village in each sub-district were calculated and each village in the selected sub-district was assigned to a quartile. Then one village was randomly selected from each quartile within the subdistrict (using syntax RANDBETWEEN in Microsoft Excel 2013, Microsoft Corporation, USA). Thus, 4 villages were selected from each of the 8 selected subdistricts in the Chittagong district, and 5 villages were selected from each of the 2 selected subdistricts in the Cox's Bazaar district.

We aimed to sample at least 123 backyard chicken farms, of which 99 also raised ducks. Therefore, in each of the 42 selected villages, 3 to 4 farms (of which 2 to 3 had to raise both chickens and ducks) needed to be recruited. Starting from the village entrance, we counted farms as we walked through the village, and recruited farms matching randomly generated numbers that were produced before the field visit. If a selected farm owner was not available or had an insufficient number of birds to be sampled, the neighbouring farm was used as a replacement. Following this procedure, we selected 144 backyard farms of which 102 also raised ducks.

2.2.2.3 | Selection of commercial chicken farms

For each selected sub-district, a sampling frame of commercial broiler and layer farmers was generated through consultations with subdistrict livestock officers, feed and chick dealers, veterinary pharmaceutical representatives, private veterinarians, feed company representatives and hatchery representatives. Information about the flock sizes of those farms was not available.

Then, simple random sampling (using syntax RANDBETWEEN in Microsoft Excel 2013, Microsoft Corporation, USA) was used to select broiler or layer farmers within each sub-district. To sample at least 103 broiler farms, 10-13 farms were randomly selected in each of the 10 selected subdistricts. In order to sample at least 102 layer farms, 10-11 farms were required per subdistrict. As only six and eight layer farms were identified in two subdistricts, all of those farms were selected in these two sub-districts, and 10-15 farms were selected from the other eight sub-districts.

2.2.2.4 | Selection of birds

As backyard chickens and in-contact ducks are free-ranging, birds were conveniently recruited by the backyard flock owner capturing available birds until the sample size was reached. A total of 4 chickens and 2 in-contact ducks were selected from backyard farms that had both, chickens and ducks, and 4 chickens were selected from farms that had chickens only.

For commercial farms, chickens were selected from different parts of the poultry shed until 8 layer and 9 broiler chickens were obtained. Selection of birds was purely based on their location within a shed and not influenced by the bird's appearance (e.g. clinical signs, plumage colour, body weight etc.). If several sheds were present on a commercial farm, the shed with oldest birds was selected

assuming that these birds had a higher chance of being exposed to avian influenza viruses throughout their production cycle.

2.3 | Sample collection and processing

Informed written consent (signature or thumb impression) was provided by each farmer before sampling of the birds and before commencement of the interview. A blood sample, a cloacal and an oro-pharyngeal swab were collected from each bird, and bird's age, sex and apparent clinical signs (if any) were recorded. Depending on the body weight, 1-3 ml blood were collected from the wing or jugular vein of each bird and transferred to individual sterile plastic tube immediately after collection. Oro-pharyngeal swabs were taken by gently rolling the swab tip around the inside of the bird's mouth and behind the tongue. Cloacal swab were collected by inserting the swab into the cloaca and rotating it several times. Swabs were placed into separate cryovials containing viral transport media. Tubes and cryovials collected in the Chittagong District were kept in a cool box filled with ice packs and transported to the Chattogram (previously Chittagong) Veterinary and Animal Sciences (CVASU) laboratory within the same day. All cryovials were stored at -80^{0}C. Blood samples were refrigerated overnight, then the serum was separated by centrifugation at 10,000 rpm for 30 minutes at 4^{0}c and transferred into Eppendorf tubes. The serum was stored at -20^{0}c until further processing. In Cox's Bazaar, samples were transported immediately to the local office of the Department of Livestock Services. Blood samples were processed as indicated above, while cryovials were stored in liquid nitrogen for up to 8 days, before being transported and stored in a -80^{0}C freezer at CVASU.

2.4 | Diagnostic tests

2.4.1 | Serological tests

The serum samples were first screened for the presence of antibodies against Influenza A virus using a commercially available Enzyme Linked Immunosorbent Assay (ELISA). For backyard chicken and duck samples, the IDEXX® AI MultiS-Screen ELISA (product code: 5004.20, IDEXX Laboratories, Inc.,USA) was used (chicken: sensitivity 98.0%, specificity 99.3%; duck: sensitivity 87.5%, specificity 98.9%). For, commercial chicken samples, either the ID Screen® Influenza A Antibody Competition Multi-Species ELISA (product Code: FLUACA ver 1216 GB, ID.vet, FRANCE) (sensitivity 98.7%, specificity 98.7%) or the IDEXX® AI ELISA (product code: 5004.00, IDEXX Laboratories, Inc.,USA)

were used (sensitivity 100.0%, specificity 99.6%). We used manufacturer recommended cut-off values to consider the sample antibody positive for Influenza A. Thus, for the IDEXX® AI MultiS-Screen ELISA, a sample was considered to be antibody positive for Influenza A if the Sample to Negative (S/N) ratio was <0.50, whereas a sample tested by ID Screen® Influenza A Antibody Competition Multi-Species ELISA was considered Influenza A antibody positive if the S/N ratio was ≤ 0.45. A sample tested by IDEXX® AI ELISA, was considered antibody positive, if the Sample to Positive (S/P) ratio was >0.50.

The Influenza A positive samples were then tested for the presence of H5 and H9 specific antibodies using the Haemagglutination Inhibition (HI) test. Inactivated antigens prepared by the Animal and Plant Health Agency in Surrey, United Kingdom were used in the HI test (H5N1-A/Ck/Scot/59, H5N3-A/Teal/Eng/7394-2805/06, H9N2-A/Tky/Wisc/1/66, H9N9-A/knot/Eng/SV497/02). A serum sample was considered H5 and H9 antibody positive if there was an inhibition at a dilution of 1/16 (2^4) or more against 4 Haemagglutinating units of antigen (OIE, 2015).

2.4.2 | Virological tests

Swab samples were pooled at the CVASU laboratory with respect to their type (cloacal and oropharyngeal) and bird species, with a maximum of five samples per pool. RNA was extracted from the pooled samples using the MagMax™-96 extraction kit (Ambion Life Technologies Corporation®, 2013). Real-time Reverse Transcription Polymerase Chain Reaction (real-time RT-PCR) was used to identify the presence of Influenza A virus by targeting the Matrix gene (M-gene). For all M-gene positive pools, RNA was extracted from the corresponding individual samples and tested by real-time RT-PCR for H5 and H9 genes (AAHL, 2014). A C_t (cycle of threshold) value <40 was considered as Influenza A virus (M-gene) positive, whereas for H5 and H9 subtypes the cut-off values were $C_t <38$ and $C_t <40$, respectively (Heine et al., 2015; Heine, Trinidad, Selleck, & Lowther, 2007; Monne et al., 2008). A bird was considered RNA virus positive, if either it's cloacal or oropharyngeal swab or if both swabs were positive.

2.5 | Data analyses

Laboratory test results were entered into Microsoft Excel 2013 spreadsheets, coded and checked for integrity, with the final dataset exported into STATA 14.1 (Stata Corporation, College Station, Texas, USA) for further statistical analysis.

2.5.1 | Bird and flock-level prevalence

Bird and flock-level apparent virus prevalence were calculated separately for Influenza A, H5 and H9. A flock was positive for a specific serological or virological test if at least one of its birds tested positive. The 95% logit confidence intervals (Dean & Pagano, 2015) were calculated for prevalence values using the -*prop*- command in STATA 14.1. If the prevalence was zero, the 97.5% binomial exact or Clopper-Pearson confidence interval (Clopper & Pearson, 1934; Dean & Pagano, 2015) was calculated using the *–cii prop*- command in STATA 14.1. To describe infection patterns over the duration of a production cycle, the bird-level apparent antibody prevalence was stratified by age groups and presented with 95% confidence intervals.

Considering the sensitivity and specificity of the HI test (Comin, Toft, Stegeman, Klinkenberg, & Marangon, 2013), the true bird-level H5 and H9 antibody prevalence with 95% confidence intervals (Blaker, 2000; Reiczigel, Földi, & Ózsvári, 2010) was calculated. The true prevalence was estimated as described by Rogan and Gladen (1978):

$$\hat{p} = \frac{\hat{t} + \beta - 1}{\alpha + \beta - 1}$$

Where, $\hat{p}$ = true prevalence, $\hat{t}$ = apparent prevalence, α = sensitivity, β = specificity

True prevalence calculations were conducted in R (R Core Team, 2017) using the epiR package (Stevenson et al., 2017).

The proportion of antibody positive backyard chickens versus antibody positive backyard in-contact ducks and the proportion of antibody positive broiler chickens versus antibody positive unvaccinated layer chickens were compared using the Fisher's Exact test (Fisher, 1935).

2.5.2 | Relationship between bird-level and flock-level antibody prevalence

We estimated the correlation between the serological antibody status of individual birds sampled within a flock (Shrout & Fleiss, 1979) by computing the individual intra-class correlation (ICC):

$$\rho = ICC = Corr(y_{ij}, y_{ij'}) = \frac{\sigma_r^2}{\sigma_r^2 + \sigma_\epsilon^2}$$

Where, σ_r^2= variance between flocks and σ_ϵ^2= error variance or variance within flocks

In our study, individual chickens (backyard, commercial broiler, layer) and in-contact ducks were considered as "raters" for the serological antibody status of a flock (represented as "target") in a one-way random effects model:

$$y_{ij} = \mu + r_i + \epsilon_{ij}$$

where y_{ij} is the jth rating on the ith target (where i= 1,…, n; j=1,…, k); μ is the mean rating; r_i is the target random effect and ϵ_{ij} is the random error (StataCorp., 2019).

2.5.3 | Spatial clusters for H5/H9 antibody positivity of backyard farms

To explore spatial patterns in viral transmission, we assessed whether H5/H9 antibody positive birds were clustered across the two study districts. The total number of antibody positive and antibody negative birds on each farm were used as the outcomes of a discrete Bernoulli probability model implemented in the SaTScan software version 9.4.4 (SaTScan™, 2016, Boston, USA). The longitudes and latitudes of the visited backyard farms were used as spatial information in the analysis. Spatial clusters of infection were identified based on 999 Standard Monte Carlo replications. The maximum size of a spatial cluster was 25% of the population at risk (Kulldorff, 1997). The analysis was conducted separately for backyard chickens and in-contact ducks, and for both, H5 and H9 subtypes.

3 | RESULTS

A total of 576 backyard chickens and 204 in-contact ducks were sampled across 144 backyard flocks, and a total of 954 broiler and 904 layer chickens were sampled from 106 broiler and 113 layer chicken flocks. None of the sampled backyard and broiler flocks, but 13 layer flocks were vaccinated against H5. HPAI outbreaks or mass mortality events were not reported in any of the backyard and commercial farms in the 12 months preceding the sampling.

The average (minimum, maximum) flock size of sampled backyard poultry, commercial broiler, unvaccinated and vaccinated commercial layer flocks were 21 (5, 73), 1,657 (200, 6,000), 2,118 (60, 7,500) and 2,831 (975, 10,500), respectively.

3.1 | Bird-level virus prevalence

Influenza A virus prevalence was 0.2% (1/576) (95% CI: 0.0-1.2%) for backyard chickens, 1% (2/204) (95% CI: 0.2-3.9%) for backyard in-contact ducks, 1.8% (17/954) (95% CI: 1.1-2.8%) for broiler chickens, 1.6% (13/800) (95% CI: 0.9-2.8%) for unvaccinated layer and 1.9% (2/104) (95% CI: 0.5-7.4%) for vaccinated layer chickens (Figure 1). None of the sampled birds on backyard and commercial farms was H5 virus RNA positive (Figure 1).

On backyard farms, 0.2% (1/576) (95% CI: 0.0-1.2%) of chickens, but none of the in-contact ducks were H9 virus RNA positive (Figure 1). Similarly, low bird-level H9 virus RNA prevalence was observed for broiler and unvaccinated commercial layer chickens, with 0.5% (5/954) (95% CI: 0.2-1.3%) and 0.6% (5/800) (95% CI: 0.3-1.5%), respectively.

3.2 | Flock-level virus prevalence

The flock-level Influenza A virus prevalence was 0.7% (1/144) (95% CI: 0.1-4.9%) for backyard flocks, 7.5% (8/106) (95% CI: 3.8-14.5%) for broiler flocks, 5.0% (5/100) (95% CI: 2.1-11.6%) for unvaccinated layer and 7.7% (1/13) (95% CI: 1.0-41.6%) for vaccinated layer flocks (Figure 1).

Relatively more unvaccinated commercial flocks were H9 virus RNA positive compared to backyard flocks, with only 0.7% (1/144) (95% CI: 0.1-4.9%) of backyard flocks, but 1.9% (2/106) (95% CI: 0.5-7.4%) and 2.0% (2/100) (95% CI: 0.5-7.8%) of broiler and unvaccinated layer flocks, respectively being H9 RNA positive (Figure 1).

3.3 | Bird-level antibody prevalence

Bird-level Influenza A antibody prevalence was 71.7% (413/576) (95% CI: 67.9-75.2%) for backyard chickens, 75.5% (154/204) (95% CI: 69.1-80.9%) for backyard in-contact ducks, 9.3% (89/954) (95% CI: 7.6-11.3%) for broiler chickens, 33.1% (265/800) (95% CI: 29.9-36.5%) for unvaccinated layer chickens, and 69.2% (72/104) (95% CI: 59.7-77.4%) for vaccinated layer chickens (Figure 2).

In backyard chickens, bird-level H5 apparent antibody -prevalence was lower compared to H9 apparent antibody prevalence - it was 4.2% (24/576) (95% CI: 2.8-6.1%) and 16.0% (92/576) (95% CI: 13.2-19.2%), respectively; while bird-level H5 and H9 apparent antibody prevalence were similar in in-contact ducks, with 14.2% (29/204) (95% CI: 10.0-19.8%) and 15.7% (32/204) (95% CI: 11.3-21.4%), respectively (Figure 2). The proportion of antibody positive backyard chickens versus antibody positive backyard in-contact ducks differed significantly for H5 antibodies ($p<0.001$), but not for H9 antibodies ($p>0.05$).

In broiler chickens, bird-level apparent antibody prevalence was 1.5% (14/954) (95% CI: 0.9-2.5%) and 1.5% (14/954) (95% CI: 0.9-2.5%) for H5 and H9, respectively. In unvaccinated layer chickens, bird-level apparent antibody prevalence was 7.8% (62/800) (95% CI: 6.1-9.8%) for H5 and 5.8% (46/800) (95% CI: 4.3-7.6%) for H9, while in vaccinated layer chickens H5 apparent antibody prevalence was 10.6% (11/104) (95% CI: 5.9-18.2). The proportion of antibody positive broiler chickens versus antibody positive unvaccinated layer chickens differed significantly for H5 antibodies ($p<0.001$) and H9 antibodies ($p<0.001$).

The estimated bird-level H5 and H9 true antibody prevalence in backyard and commercial chickens is shown in Table 1. Considering the high sensitivity (98.8%) and specificity (99.5%) of the HI test, there was no considerable discrepancy between the apparent prevalence derived from the test results only and the true prevalence considering the test characteristics.

3.4 | Flock-level antibody prevalence

The flock-level Influenza A antibody prevalence was 97.2% (140/144) (95% CI: 92.8-99.0%) for backyard flocks, 17.9% (19/106) (95% CI: 11.7-26.6%) for broiler flocks, 52.0% (52/100) (95% CI:

42.1-61.7%) for unvaccinated layer flocks, and 84.6% (11/13) (95% CI: 53.0-96.4%) for vaccinated layer flocks (Figure 2).

In backyard poultry, flock-level antibody prevalence was 27.8% (40/144) (95% CI: 21.0-35.7%) for H5 and 60.4% (87/144) (95% CI: 52.1-68.2%) for H9 antibodies (Figure 2). In contrast, flock-level H5 antibody prevalence was higher than H9 antibody prevalence in broiler and unvaccinated layers flocks: it was 9.4% (10/106) (95% CI: 5.1-16.8%) and 5.7% (6/106) (95% CI: 2.5-12.2%) in broilers and 31.0% (31/100) (95% CI: 22.6-40.9%) and 22.0% (22/100) (95% CI: 14.9-31.3%) in unvaccinated layer flocks. The flock-level H5 antibody prevalence in vaccinated layer flocks was 38.5% (5/13) (95% CI: 16.2-66.9%).

3.5 | Relationship between bird-level and flock-level antibody prevalence

In 70.0% of H5 antibody positive and in 66.7% of H9 antibody positive backyard flocks, only single birds (either chickens or ducks) tested positive within the flock. Interestingly, in only 5.0% of backyard flocks, both chickens and ducks were found to be H5 antibody positive, while in 13.8% of backyard flocks, both chickens and ducks were found to be H9 antibody positive.

The clustering effect of birds being antibody positive within a flock was represented by the ICC (Figure 3). As usually only single chickens were H5 or H9 antibody positive within backyard flocks, the ICC was low for backyard chickens (H5: ICC=0.07, 95% CI: 0.0-0.2; H9: ICC=0.04, 95% CI: 0.0-0.1). In contrast, often multiple ducks with a backyard flock were antibody positive (H5: ICC=0.48, 95% CI: 0.3-0.6; H9: ICC=0.19, 95% CI: 0.0-0.4).

All broiler flocks had only 1-2 chickens that tested H5 antibody positive across the 9 birds sampled per flock (H5: ICC=0.06, 95% CI: 0.0-0.1), whereas for H9 antibodies, about half of the broiler flocks had 1-2 birds, and the other half had 3-4 birds, that tested positive (H9: ICC=0.22, 95% CI: 0.2-0.3). As similar low clustering effect for H5 and H9 antibody positivity was observed for unvaccinated layer flocks with 1-2 birds of the 8 birds sampled tested H5 antibody positive in 71% of flocks and with 1-2 birds of the 8 birds sampled tested H9 antibody positive in 73% of flocks (H5: ICC=0.15, 95% CI: 0.1-0.2; H9: ICC=0.17, 95% CI: 0.1-0.2). Surprisingly, in 60% of vaccinated layer flocks only 1-2 birds tested H5 antibody positive (layer H5: ICC=0.19, 95% CI: 0.1-0.5).

3.6 | Antibody prevalence by age groups

Over the course of a production cycle, higher bird-level H5 and H9 antibody prevalence was, in general, observed in older backyard chickens and ducks as well as in older unvaccinated layers (Figure 4). Interestingly, H5 antibody prevalence peaked around 1.5 years in backyard chickens and in unvaccinated layers, but then declined afterwards. A similar decline in older birds was not observed for H9 antibody positivity in backyard chickens and unvaccinated layers.

An increase in H5 and H9 titres with age was not so prominent in broilers.

Surprisingly, H5 titres in vaccinated layers were low in the first year of age (when vaccination of layers is conducted) and only peaked at 1.5 years of age and drastically declined afterwards.

3.7 | Spatial clusters for H5/H9 antibody positivity of backyard farms

The relatively high flock-level antibody prevalence of backyard flocks and a strong clustering of ducks being antibody positive within backyard flocks, intrigued us to further explore the spatial distribution of H5 and H9 antibody positivity of backyard poultry within our study area.

When analysing the locations of chickens being positive within backyard farms, a high-risk cluster for H5 antibody positivity (RR=5.4, p=0.004, radius=16.8 km) and a spatially overlapping high-risk cluster for H9 antibody positivity (RR=15.2, p=0.036, radius=15.2 km) were identified in the central part of the Chittagong district (Figure 5). This area is represented by high densities of backyard poultry farms, proximity to the Chittagong city (where most live bird markets are located), and importantly, the largest river in the Chittagong district, the Karnaphuli river, passes through the clusters.

Interestingly, when locations of ducks being positive within a backyard farm were analysed, only a small high risk cluster for H9 (RR=7.7, p=0.048, radius=0.6 km) was identified, highlighting that the risk of ducks being H5 and H9 antibody positive was uniform throughout the study area (Figure 6).

4 | DISCUSSION

This is the first study comprehensively investigating the extent of H5 and H9 virus circulation among populations of backyard, commercial broiler and layer chickens in a H5N1-endemic country.

Recently, avian influenza virus prevalence was estimated for Bangladeshi LBMs, including LBMs in Chittagong City, which are supplied by poultry farms located in our study area (Kim et al., 2018). H5 and H9 virus prevalence on markets was 1.3% and 8.3 % in backyard chickens, 7.6% and 3.4% in waterfowl (including ducks and geese), 0.9% and 13.1% in broiler chickens, respectively (Kim et al., 2018). In contrast, all birds in our study were H5 virus RNA negative and H9 virus RNA prevalence on our poultry farms was much lower compared to the LBMs investigated by Kim et al., 2018. This may be due to the amplification of the avian influenza virus along the trading networks from farms to LBMs or at the LBMs themselves (Kung et al., 2007).

Interestingly, the proportion of flocks positive for the Influenza A (M-gene) and for H9 virus RNA was similar for broiler and layer farms. This suggests that the level of exposure of broiler flocks to avian influenza viruses may be similar to layer flocks. Although avian influenza vaccination programmes usually focus on layer farms, our results indicate that vaccinations of broilers should be considered as well. However, a detailed cost-benefit analysis is required before such an avian influenza prevention strategy could be implemented for broiler flocks.

We used serological and virological testing to evaluate the infection status of birds. As infected birds may shed avian influenza viruses only for 3-7 days, serological testing is useful to assess the past infection status of birds (Achenbach & Bowen, 2011; Leigh Perkins & Swayne, 2002; Saito et al., 2009; Spackman, Pantin-Jackwood, Swayne, & Suarez, 2009; Sturm-Ramirez et al., 2004). For example, it has been shown that H5 antibodies persist up to 40 weeks post-vaccination in H5N3 vaccinated chickens and ducks (Boltz et al., 2009), while H9N2 antibodies had been detected for up to 15 weeks in unvaccinated chickens (Imai et al., 2007).

In our study, H5 bird-level antibody prevalence was higher in ducks than in chickens on backyard farms. Such patterns were also described for backyard poultry populations in Vietnam and Indonesia (Henning et al., 2011; Henning et al., 2010). One plausible explanation is that chickens infected by HPAI H5 will most likely die, whereas ducks may survive (Kishida et al., 2005), although

farmers in our study did not report any significant increase in chicken mortality over the year preceding the sampling. Other possible explanations for the higher antibody prevalence in ducks are that antibodies might persist for longer in ducks, or that ducks might be exposed more frequently to H5 viruses as they are frequently mingle with (potentially infected) wild waterfowls on water bodies (Hill et al., 2015; Khatun et al., 2013). Thus, as suggested previously, ducks may be a major source of H5 virus infection for backyard chickens and other poultry (Henning et al., 2011; Hulse-Post et al., 2005; Kishida et al., 2005; Sarkar et al., 2017).

Whereas previous studies (Ansari et al., 2016; Karki, Lupiani, Budke, Manandhar, & Ivanek, 2014; Khatun et al., 2013) generally only reported bird-level prevalence, we estimated the clustering effects of birds being antibody positive within a flock. Our results indicated that usually multiple ducks in a flock are antibody positive, which is in contrast to backyard chickens and broiler and layer chicken flocks, where only single birds within these flocks developed H5 antibodies.

Similarly to a study on ducks in Vietnam (Henning et al., 2011), H5 and H9 antibody prevalence increased with the age of backyard and layer birds. Indeed, it is expected that the likelihood of multiple exposures to endemic viruses increases with a higher age of birds. However, we did not observe the same magnitude of increased H5 antibody prevalence in broilers compared to layers and backyard chickens. Considering the short production cycle of broilers there are reduced opportunities to observe a significant rise of antibody titres. In fact, field research had highlighted that avian influenza antibodies (H9) could only be observed 2 weeks past infection (Nili & Asasi, 2002)**,** highlighting that due to the shorter lifespan of broilers, there are limitations in monitoring an immune response in broilers. We also did not find any H5 antibodies in very young broiler and layer chicks indicating that no maternal antibodies from the vaccinated breeder or parent birds were detectable in this age group (based on Bangladeshi government regulations breeder or parent stock of broilers and layers should be vaccinated against H5, DLS (2013)).

Interestingly no major mortalities or clinical HPAI symptoms were observed in backyard and commercial chicken flocks, although birds did developed antibodies. The H5 antibodies detected might have resulted from infection with LPAI H5 strains, which would not caused deaths of birds. A number of studies reported LPAI H5 viruses (H5N2, H5N3, H5N8) occurring in Asia (Duan et al., 2007;

Nguyen et al., 2005), including LPAI H5N2 virus in Bangladesh (Gerloff et al., 2016). Another explanation might be a reduction in H5N1 pathogenicity due to viral evolution (Li et al., 2017; Londt, Banks, & Alexander, 2007) and the development of cell-mediated immunity that contributes to host resistance (Kapczynski, 2008; Wang, Loh, Kedzierski, & Kedzierska, 2016).

In Bangladesh, two inactivated vaccines are used for commercial layers and parent stocks: (1) *Re-6* from Merial (produced in China), containing the HA gene from a clade 2.3.2.1 H5N1 virus, and (2) *Nobilis Influenza H5*, an inactivated H5N2 vaccine from Intervet (produced in the Netherlands). In addition, one live vector vaccine, *Vectormune HVT-AIV* from CEVA-Biomine (produced in the USA), comprising of an innocent vector virus containing HA gene of AI virus, is used for vaccination of day-old layer and broiler chicks (this vaccine is endorsed by the Bangladeshi government and is used in hatcheries). Surprisingly, we found that a substantial proportion of vaccinated layer chickens did not developed an immune response. This limited immune response might be due improper vaccination procedures or poor vaccine quality. However, higher bird-level H5 antibody prevalence was observed in older vaccinated birds, which might be due to repeated vaccinations, or exposure to LPAI H5 field viruses.

Our spatial cluster analysis revealed consistent H5 (past) infection of ducks across the whole study area. In contrast, for backyard chickens, H5 and H9 antibody positive cases were clustered in a specific location, with the Karnaphuli River, the largest river in the Chittagong district, passing through this cluster. Indeed, river systems in Bangladesh have been hypothesized to represent high-risk areas for HPAI H5N1 infection, although no biological sampling of birds had been conducted in such river systems (Ahmed et al., 2012; Muzaffar et al., 2008).

There are some limitations in our study. Firstly, the HI test antigens were prepared from field virus isolated from a range of countries, but not from a field virus collected in Bangladesh. However, a study conducted by Yamamoto et al. (2007) estimated sensitivity and specificity of the HI test to be 99% and 90%, respectively, when different antigens were used. Considering this good specificity of the HI test using different antigens, we are confident that our estimated antibody prevalence was not overestimated due to many false-positive results.

Secondly, recall bias may have led to an incorrect estimation of the age of chickens. This would only relate to backyard farmers, as commercial flock owners usually record the dates when they start a production cycle with day-old chicks. Unfortunately, dates of vaccinations were not recorded by layer farmers, and therefore we were unable to relate antibody titres observed to the timing of farm-specific vaccination programmes.

Finally, we conducted this study in two districts of Bangladesh and, although these districts represent administrative areas with a significant poultry population, our research findings might not be able to be extrapolated to the entire country of Bangladesh. Also due to the cross-sectional nature of our study design we were not able to describe seasonal variations in avian influenza infection patterns.

In conclusion, this research provided unique insights into current and past H5 and H9 infection pattern across all chicken production systems in the Chittagong and Cox's Bazaar districts of Bangladesh.

Data generated in this research can be used to adopt risk-based surveillance approaches adjusted to the expected risk of infection in different chicken production systems in Bangladesh. In addition, the estimated avian influenza prevalence in farmed poultry populations can inform mathematical models exploring HPAI infection dynamics commencing at the source of poultry production.

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ETHICS STATEMENT

The authors confirm that the ethical policies of the journal, as noted on the journal's author guidelines page, have been adhered to and the appropriate ethical review committee approval has been received. Animal (Approval Number: SVS/465/15/RVC) and Human (Approval Number: 2015001703) Ethics approval for the research was obtained from the relevant ethics committees at the University of Queensland.

CONFLICT OF INTEREST

None

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

REFERENCES

AAHL. (2014). *Molecular Diagnosis at CSIRO Animal, Food and Health Science-Nucleic acid detection for disease diagnosis and emergency disease investigation- H5, H9 TaqManR Assays*- AAHL Regional Programme, December, 2014.

Achenbach, J. E., & Bowen, R. A. (2011). Transmission of Avian Influenza A Viruses among Species in an Artificial Barnyard. *PLoS ONE, 6*(3), e17643. https://doi.org/10.1371/journal.pone.0017643

Ahmed, S. S., Ersbøll, A. K., Biswas, P. K., Christensen, J. P., Hannan, A. S., & Toft, N. (2012). Ecological determinants of highly pathogenic avian influenza (H5N1) outbreaks in Bangladesh. *PLoS ONE, 7*(3), e33938. https://doi.org/10.1371/journal.pone.0033938

Alam, M., Ali, M., Das, N., & Rahman, M. (2014). Present status of rearing backyard poultry in selected areas of Mymensingh district. *Bangladesh Journal of Animal Science, 43*(1), 30-37. https://doi.org/10.3329/bjas.v43i1.19382

Alexander, D. J. (2000). A review of avian influenza in different bird species. *Veterinary Microbiology, 74*(1-2), 3-13. https://doi.org/10.1016/S0378-1135(00)00160-7

Ansari, W. K., Parvej, M. S., El Zowalaty, M. E., Jackson, S., Bustin, S. A., Ibrahim, A. K., . . . Ahamd, M. M. (2016). Surveillance, epidemiological, and virological detection of highly pathogenic H5N1 avian influenza viruses in duck and poultry from Bangladesh. *Veterinary Microbiology*. https://doi.org/10.1016/j.vetmic.2016.07.025

Barua, A., & Yoshimura, Y. (1997). Rural poultry keeping in Bangladesh. *World's Poultry Science Journal, 53*(04), 387-394. https//doi.org/10.1079/WPS19970031

BBS. (2011a). *Census of Agriculture 2008, Zila Series: Chittagong*. The Bangladesh Bureau of Statistics(BBS), Statistics and informatics division, Ministry of planning, Government of the People's Republic of Bangladesh.

BBS. (2011b). *Census of Agriculture 2008, Zila Series: Cox's Bazaar*. The Bangladesh Bureau of Statistics(BBS), Statistics and informatics division, Ministry of planning, Government of the People's Republic of Bangladesh.

BBS. (2011c). *Population & housing census, national Vol. 3, urban area report*. The Bangladesh Bureau of Statistics(BBS), Statistics and informatics division, Ministry of planning, Government of the People's Republic of Bangladesh.

BBS. (2014). *Bangladesh Population and Housing Census 2011, Community Report, Zila: Cox's Bazar*. The Bangladesh Bureau of Statistics(BBS), Statistics and informatics division, Ministry of planning, Government of the People's Republic of Bangladesh. Retrieved from https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=2ahUKEwjAgPT9gLrkAhXeIbcAHWmBAkAQFjAAegQIAhAC&url=http%3A%2F%2F203.112.218.65%3A8008%2FWebTestApplication%2Fuserfiles%2FImage%2FPopCen2011%2FCom_Cox%2527s%2520Bazar.pdf&usg=AOvVaw2KxgYROo95tLqyjktZhitZ.

BBS. (2015). *Bangladesh Population and Housing Census 2011, Community Report, Zila: Chittagong*. The Bangladesh Bureau of Statistics(BBS), Statistics and informatics division, Ministry of planning, Government of the People's Republic of Bangladesh. Retrieved from https://www.google.com/url?sa=t&rct=j&q=&esrc=s&source=web&cd=1&ved=2ahUKEwia-Y2VgLrkAhUFiOYKHXt8CpUQFjAAegQIABAC&url=http%3A%2F%2F203.112.218.65%3A8008%2FWebTestApplication%2Fuserfiles%2FImage%2FPopCen2011%2FCom_Chittagong.pdf&usg=AOvVaw3F7Q-oaJcIaZ8oPcgwoa3F.

Biswas, P. K., Christensen, J. P., Ahmed, S. S., Das, A., Rahman, M. H., Barua, H., . . . Debnath, N. C. (2009). Risk for infection with highly pathogenic avian influenza virus (H5N1) in backyard chickens, Bangladesh. *Emerging Infectious Diseases, 15*(12), 1931-1936. https://dx.doi.org/10.3201%2Feid1512.090643

Blaker, H. (2000). Confidence curves and improved exact confidence intervals for discrete distributions. *Canadian Journal of Statistics, 28*(4), 783-798. https://dx.doi.org/10.2307/3315916

Boltz, D. A., Douangngeun, B., Sinthasak, S., Phommachanh, P., Midouangchanh, P., Walker, D., . . . Webster, R. G. (2009). Field assessment of an H5N1 inactivated vaccine in chickens and ducks in Lao PDR. *Archives of Virology, 154*(6), 939-944. https://dx.doi.org/10.1007%2Fs00705-009-0385-x

CDC. (2019). *Highly Pathogenic Asian Avian Influenza A(H5N1) Virus*. Retrieved from https://www.cdc.gov/flu/avianflu/h5n1-virus.htm

Chattopadhyay, K., Fournié, G., Kalam, M. A., Biswas, P. K., Hoque, A., Debnath, N. C., . . . Heymann, D. L. (2018). A qualitative stakeholder analysis of avian influenza policy in Bangladesh. *EcoHealth, 15*(1), 63-71. https://doi.org/10.1007/s10393-017-1285-2

Clopper, C. J., & Pearson, E. S. (1934). The use of confidence or fiducial limits illustrated in the case of the binomial. *Biometrika, 26*(4), 404-413.

Comin, A., Toft, N., Stegeman, A., Klinkenberg, D., & Marangon, S. (2013). Serological diagnosis of avian influenza in poultry: is the haemagglutination inhibition test really the 'gold standard'? *Influenza and other Respiratory Viruses, 7*(3), 257-264. https://doi.org/10.1111/j.1750-2659.2012.00391.x

Dean, N., & Pagano, M. (2015). Evaluating Confidence Interval Methods for Binomial Proportions in Clustered Surveys. *Journal of Survey Statistics and Methodology, 3*(4), 484-503. https://doi.org/10.1093/jssam/smv024

DLS. (2013). Experimental vaccination against HPAI in selected areas of Bangladesh. The Department of Livestock Services (DLS), Ministry of Fisheries and Livestock, Government of the People's Republic of Bangladesh.DLS. (2014). *Poultry farm statistics (as of September, 2014)*. The Department of Livestock Services (DLS), Ministry of Fisheries and Livestock, Government of the People's Republic of Bangladesh.

DLS. (2019). *HPAI outbreaks report: 2017-2019(as of August 2019)*. The Department of Livestock Services (DLS), Ministry of Fisheries and Livestock, Government of the People's Republic of Bangladesh.

Duan, L., Campitelli, L., Fan, X., Leung, Y., Vijaykrishna, D., Zhang, J., . . . Foni, E. (2007). Characterization of low-pathogenic H5 subtype influenza viruses from Eurasia: implications for the origin of highly pathogenic H5N1 viruses. *Journal of Virology, 81*(14), 7529-7539. https://doi.org/10.1128/JVI.00327-07

ElMasry, I., Elshiekh, H., Abdlenabi, A., Saad, A., Arafa, A., Fasina, F. O., . . . Jobre, Y. (2017). Avian influenza H5N1 surveillance and its dynamics in poultry in live bird markets, Egypt. *Transboundary and Emerging Diseases, 64*(3), 805-814. https://doi.org/10.1111/tbed.12440

FAO. (2008). *Bangladesh poultry sector country review*. Retrieved from www.fao.org/3/a-ai319e.pdf

FAO. (2011). *Approaches to controlling, preventing and eliminating H5N1 Highly Pathogenic Avian Influenza in endemic countries*. Retrieved from http://www.fao.org/3/i2150e/i2150e00.htm

Fisher, R. A. (1935). The logic of inductive inference. *Journal of the Royal Statistical Society, 98*(1), 39-82.

Fournié, G., Høg, E., Barnett, T., Pfeiffer, D. U., & Mangtani, P. (2017). A Systematic Review and Meta-Analysis of Practices Exposing Humans to Avian Influenza Viruses, Their Prevalence, and Rationale. *The American Journal of Tropical Medicine and Hygiene, 97*(2), 376-388. https://doi.org/10.4269/ajtmh.17-0014

Gerloff, N. A., Khan, S. U., Zanders, N., Balish, A., Haider, N., Islam, A., . . . Hosseini, P. (2016). Genetically diverse low pathogenicity avian influenza A virus subtypes co-circulate among poultry in Bangladesh. *PLoS ONE, 11*(3), e0152131. https://doi.org/10.1371/journal.pone.0152131

Haider, N., Sturm-Ramirez, K., Khan, S. U., Rahman, M. Z., Sarkar, S., Poh, M. K., . . . Zeidner, N. (2015). Unusually High Mortality in Waterfowl Caused by Highly Pathogenic Avian Influenza A(H5N1) in Bangladesh. *Transboundary and Emerging Diseases*, *64*(1), 144-156. https://doi.org/10.1111/tbed.12354

Haque, M. E., Kabir, M. L., Ali, M. M., Rahman, H. H., & Islam, M. A. (2015). Co-circulation of three sub-types of avian influenza viruses in the poultry population of Bangladesh in the year 2014. *Trends in Life Sciences, 4*(1), 2319–5037.

Hassan, M. M., & Nazem, M. N. I. (2016). Examination of land use/land cover changes, urban growth dynamics, and environmental sustainability in Chittagong city, Bangladesh. *Environment, Development and Sustainability, 18*(3), 697-716. https://doi.org/10.1007/s10668-015-9672-8

Hassan, M. M. M. (2017). *Who is the culprit: ecology and epidemiology of Avian Influenza at the wildlife-poultry interface in Bangladesh.* (Doctoral dissertation). Retrieved from DRO (Deakin University's research repository). (PAC-10101609)

Heine, H. G., Foord, A. J., Wang, J., Valdeter, S., Walker, S., Morrissy, C., . . . Meehan, B. (2015). Detection of highly pathogenic zoonotic influenza virus H5N6 by reverse-transcriptase quantitative polymerase chain reaction. *Virology Journal, 12*(1), 18. https://doi.org/10.1186/s12985-015-0250-3

Heine, H. G., Trinidad, L., Selleck, P., & Lowther, S. (2007). Rapid Detection of Highly Pathogenic Avian Influenza H5N1 Virus by TaqMan Reverse Transcriptase–Polymerase Chain Reaction. *Avian Diseases, 51*(s1), 370-372, 373. https://doi.org/10.1637/7587-040206R.1

Henning, J., Henning, K. A., Morton, J. M., Long, N. T., Ha, N. T., Vu le, T., . . . Meers, J. (2011). Highly pathogenic avian influenza (H5N1) in ducks and in-contact chickens in backyard and smallholder commercial duck farms in Viet Nam. *Preventive Veterinary Medicine, 101*(3-4), 229-240. https://doi.org/10.1016/j.prevetmed.2010.05.016

Henning, J., Wibawa, H., Morton, J., Usman, T. B., Junaidi, A., & Meers, J. (2010). Scavenging ducks and transmission of highly pathogenic avian influenza, Java, Indonesia. *Emerging Infectious Diseases, 16*(8), 1244-1250. https://dx.doi.org/10.3201%2Feid1608.091540

Hill, S. C., Lee, Y.-J., Song, B.-M., Kang, H.-M., Lee, E.-K., Hanna, A., . . . Pybus, O. G. (2015). Wild waterfowl migration and domestic duck density shape the epidemiology of highly pathogenic H5N8 influenza in the Republic of Korea. *Infection, Genetics and Evolution, 34*, 267-277. https://doi.org/10.1016/j.meegid.2015.06.014

Hulse-Post, D., Sturm-Ramirez, K., Humberd, J., Seiler, P., Govorkova, E., Krauss, S., . . . Nguyen, T. (2005). Role of domestic ducks in the propagation and biological evolution of highly pathogenic H5N1 influenza viruses in Asia. *Proceedings of the National Academy of Sciences, 102*(30), 10682-10687. https://doi.org/10.1073/pnas.0504662102

Humphry, R. W., Cameron, A., & Gunn, G. J. (2004). A practical approach to calculate sample size for herd prevalence surveys. *Preventive Veterinary Medicine, 65*(3), 173-188. https://doi.org/10.1016/j.prevetmed.2004.07.003

Huque, K., Saleque, M., & Khatun, R. (2011). *Commercial poultry production in Bangladesh.* Paper presented at the 7th international Poultry Show and Seminar2011. World's Poultry Science Association, Bangladesh Branch.

Imai, K., Nakamura, K., Mase, M., Tsukamoto, K., Imada, T., & Yamaguchi, S. (2007). Partial protection against challenge with the highly pathogenic H5N1 influenza virus isolated in Japan in chickens infected with the H9N2 influenza virus. *Archives of Virology, 152*(7), 1395-1400. https://doi.org/10.1007/s00705-007-0953-x

Kapczynski, D. R. (2008). Evaluating the Cell-Mediated Immune Response of Avian Species to Avian Influenza Viruses. In E. Spackman (Ed.), *Avian Influenza Virus* (pp. 113-126). Totowa, NJ: Humana Press.

Karki, S., Lupiani, B., Budke, C., Manandhar, S., & Ivanek, R. (2014). Cross-Sectional Serosurvey of Avian Influenza Antibodies Presence in Domestic Ducks of Kathmandu, Nepal. *Zoonoses and Public Health, 61*(6), 442-448. https://doi.org/10.1111/zph.12097

Khatun, A., Giasuddin, M., Islam, K. M., Khanom, S., Samad, M. A., Islam, M. R., . . . Eo, S. K. (2013). Surveillance of avian influenza virus type A in semi-scavenging ducks in Bangladesh. *BMC Veterinary Research, 9*(1), 1. https://doi.org/10.1186/1746-6148-9-196

Kim, Y., Biswas, P. K., Giasuddin, M., Hasan, M., Mahmud, R., Chang, Y.-M., . . . Fournié, G. (2018). Prevalence of Avian Influenza A(H5) and A(H9) Viruses in Live Bird Markets, Bangladesh. *Emerging Infectious Diseases, 24*(12), 2309-2316. https://dx.doi.org/10.3201%2Feid2412.180879

Kishida, N., Sakoda, Y., Isoda, N., Matsuda, K., Eto, M., Sunaga, Y., . . . Kida, H. (2005). Pathogenicity of H5 influenza viruses for ducks. *Archives of Virology, 150*(7), 1383-1392. https://doi.org/10.1007/s00705-004-0473-x

Kulldorff, M. (1997). A spatial scan statistic. *Communications in Statistics-Theory and methods, 26*(6), 1481-1496. https://doi.org/10.1080/03610929708831995

Kung, N. Y., Morris, R. S., Perkins, N. R., Sims, L. D., Ellis, T. M., Bissett, L., . . . Peiris, M. J. (2007). Risk for infection with highly pathogenic influenza A virus (H5N1) in chickens, Hong Kong, 2002. *Emerging Infectious Diseases, 13*(3), 412. https://dx.doi.org/10.3201%2Feid1303.060365

Leigh Perkins, L. E., & Swayne, D. E. (2002). Pathogenicity of a Hong Kong–origin H5N1 highly pathogenic avian influenza virus for emus, geese, ducks, and pigeons. *Avian Diseases, 46*(1), 53-63. https://doi.org/10.1637/0005-2086(2002)046[0053:POAHKO]2.0.CO;2

Li, K.-P., Chang, P.-C., Cheng, M.-C., Tan, D.-H., Chen, L.-H., Liu, Y.-P., . . . Shien, J.-H. (2017). Sequence diversity and associated pathogenicity of the hemagglutinin cleavage site of H5N2 avian influenza viruses isolated from chickens in Taiwan during 2013-2015. *The Journal of Veterinary Medical Science, 79*(1), 108-114. https://doi.org/10.1292/jvms.16-0356

Londt, B. Z., Banks, J., & Alexander, D. J. (2007). Highly pathogenic avian influenza viruses with low virulence for chickens in in vivo tests. *Avian Pathology, 36*(5), 347-350. https://doi.org/10.1080/03079450701589134

Loth, L., Gilbert, M., Osmani, M. G., Kalam, A. M., & Xiao, X. (2010). Risk factors and clusters of highly pathogenic avian influenza H5N1 outbreaks in Bangladesh. *Preventive Veterinary Medicine, 96*(1), 104-113. https://doi.org/10.1016/j.prevetmed.2010.05.013

Marinova-Petkova, A., Shanmuganatham, K., Feeroz, M. M., Jones-Engel, L., Hasan, M. K., Akhtar, S., . . . Webster, R. G. (2016). The Continuing Evolution of H5N1 and H9N2 Influenza Viruses in Bangladesh Between 2013 and 2014. *Avian Diseases, 60*(1 Suppl), 108-117. https://doi.org/10.1637/11136-050815-Reg

Monne, I., Ormelli, S., Salviato, A., De Battisti, C., Bettini, F., Salomoni, A., . . . Cattoli, G. (2008). Development and Validation of a One-Step Real-Time PCR Assay for Simultaneous Detection of Subtype H5, H7, and H9 Avian Influenza Viruses. *Journal of Clinical Microbiology, 46*(5), 1769-1773. https://doi.org/10.1128/jcm.02204-07

Moyen, N. (2019). *Transmission of avian influenza viruses through live bird trade networks in Bangladesh* (unpublished doctoral dissertation). Royal Veterinary College, University of London, United Kingdom.

Moyen, N., Ahmed, G., Gupta, S., Tenzin, T., Khan, R., Khan, T., . . . Fournie, G. (2018). A large-scale study of a poultry trading network in Bangladesh: implications for control and surveillance of avian influenza viruses. *BMC Veterinary Research, 14*(1), 12. https://doi.org/10.1186/s12917-018-1331-5

Muzaffar, S., Takekawa, J. Y., Prosser, D. J., Douglas, D. C., Yan, B., Xing, Z., . . . Newman, S. H. (2008). Seasonal movements and migration of Pallas's Gulls Larus ichthyaetus from Qinghai Lake, China. *Forktail, 24*(2008), 100-107.

Negovetich, N. J., Feeroz, M. M., Jones-Engel, L., Walker, D., Alam, S. R., Hasan, K., . . . Barman, S. (2011). Live bird markets of Bangladesh: H9N2 viruses and the near absence of highly pathogenic H5N1 influenza. *PLoS ONE, 6*(4), e19311. https://doi.org/10.1371/journal.pone.0019311

Nguyen, D. C., Uyeki, T. M., Jadhao, S., Maines, T., Shaw, M., Matsuoka, Y., . . . Hall, H. (2005). Isolation and characterization of avian influenza viruses, including highly pathogenic H5N1,

from poultry in live bird markets in Hanoi, Vietnam, in 2001. *Journal of Virology, 79*(7), 4201-4212. https://doi.org/10.1128/JVI.00327-07

Nili, H., & Asasi, K. (2002). Natural cases and an experimental study of H9N2 avian influenza in commercial broiler chickens of Iran. *Avian Pathology, 31*(3), 247-252. https://doi.org/10.1080/03079450220136567

Nooruddin, G., Hossain, M., Mohammad, M., & Rahman, M. M. (2006). Sero-epidemiology of avian influenza virus in native chicken in Bangladesh. *International Journal of Poultry Science, 5*, 1029-1033.

OIE. (2015). *Manual of Diagnostic Tests and Vaccines for Terrestrial Animals*. Retrieved from https://www.oie.int/fileadmin/Home/eng/Health_standards/tahm/2.03.04_AI.pdf.

Osmani, M., Thornton, R., Dhand, N. K., Hoque, M., Milon, S., Kalam, M., . . . Yamage, M. (2014). Risk factors for highly pathogenic avian influenza in commercial layer chicken farms in Bangladesh During 2011. *Transboundary and Emerging Diseases, 61*(6), e44-e51. https://doi.org/10.1111/tbed.12071

Parvin, R., Begum, J. A., Chowdhury, E. H., Islam, M. R., Beer, M., & Harder, T. (2019). Co-subsistence of avian influenza virus subtypes of low and high pathogenicity in Bangladesh: Challenges for diagnosis, risk assessment and control. *Scientific Reports, 9*(1), 8306. https://doi.org/10.1038/s41598-019-44220-4

Parvin, R., Begum, J. A., Nooruzzaman, M., Chowdhury, E. H., Islam, M. R., & Vahlenkamp, T. W. (2018). Review analysis and impact of co-circulating H5N1 and H9N2 avian influenza viruses in Bangladesh. *Epidemiology & Infection, 146*(10), 1259-1266. https://doi.org/10.1017/S0950268818001292

Peacock, T. H. P., James, J., Sealy, J. E., & Iqbal, M. (2019). A Global Perspective on H9N2 Avian Influenza Virus. *Viruses, 11*(7), 620. https://doi.org/10.3390/v11070620

R Core Team. (2017). R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria. Retrieved from https://www.R-project.org/

Reiczigel, J., Földi, J., & Ózsvári, L. (2010). Exact confidence limits for prevalence of a disease with an imperfect diagnostic test. *Epidemiology & Infection, 138*(11), 1674-1678. https://doi.org/10.1017/S0950268810000385

Rogan, W. J., & Gladen, B. (1978). Estimating prevalence from the results of a screening test. *American Journal of Epidemiology*, *107*(1), 71-76. https://doi.org/10.1093/oxfordjournals.aje.a112510

Saito, T., Watanabe, C., Takemae, N., Chaisingh, A., Uchida, Y., Buranathai, C., . . . Yamaguchi, S. (2009). Pathogenicity of highly pathogenic avian influenza viruses of H5N1 subtype isolated in Thailand for different poultry species. *Veterinary Microbiology, 133*(1), 65-74. https://doi.org/10.1016/j.vetmic.2008.06.020

Sarkar, S., Khan, S. U., Mikolon, A., Rahman, M. Z., Abedin, J., Zeidner, N., . . . Luby, S. P. (2017). An epidemiological study of avian influenza A (H5) virus in nomadic ducks and their raising practices in northeastern Bangladesh, 2011-2012. *Influenza and other Respiratory Viruses, 11*(3), 275-282. https://doi.org/10.1111/irv.12438

Sergeant, E., & Perkins, N. (2015). *Epidemiology for field veterinarians: An Introduction*. UK:CABI.

Shrout, P. E., & Fleiss, J. L. (1979). Intraclass correlations: uses in assessing rater reliability. *Psychological bulletin, 86*(2), 420.

Spackman, E., Pantin-Jackwood, M. J., Swayne, D. E., & Suarez, D. L. (2009). An evaluation of avian influenza diagnostic methods with domestic duck specimens. *Avian Diseases, 53*(2), 276-280. https://doi.org/10.1637/8520-111708-Reg.1

Spradbrow, P. (1996). *Protection against important diseases including Newcastle disease.* Paper presented at the Proceedings of the 20th World's Poultry Congress, New Delhi, India.

StataCorp. (2019). icc — Intraclass correlation coefficients.Stata Base Reference Manual: Release 16 (p. 1033-1055): Stata Corporation, College Station, TX: Stata Press. Retrieved from https://www.stata.com/bookstore/base-reference-manual/.

Stevenson, M., Nunes, T., Heuer, C., Marshall, J., Sanchez, J., Thornton, R., . . . Kyle, R. (2017). epiR: Tools for the Analysis of Epidemiological Data.

Sturm-Ramirez, K. M., Ellis, T., Bousfield, B., Bissett, L., Dyrting, K., Rehg, J. E., . . . Webster, R. G. (2004). Reemerging H5N1 influenza viruses in Hong Kong in 2002 are highly pathogenic to ducks. *Journal of Virology, 78*(9), 4892-4901. https://doi.org/10.1128/JVI.78.9.4892-4901.2004

Thuy, D. M., Peacock, T. P., Bich, V. T. N., Fabrizio, T., Hoang, D. N., Tho, N. D., . . . Bryant, J. E. (2016). Prevalence and diversity of H9N2 avian influenza in chickens of Northern Vietnam, 2014. *Infection, Genetics and Evolution, 44*, 530-540. https://doi.org/10.1016/j.meegid.2016.06.038

Wang, Z., Loh, L., Kedzierski, L., & Kedzierska, K. (2016). Avian influenza viruses, inflammation, and CD8+ T cell immunity. *Frontiers in Immunology, 7*, 60. https://doi.org/10.3389/fimmu.2016.00060

WHO. (2020). *Cumulative number of confirmed human cases for avian influenza A(H5N1) reported to World Health Organization (WHO), 2003 to 2020 (as of January)*. Retrieved from https://www.who.int/influenza/human_animal_interface/H5N1_cumulative_table_archives/en/

Yamamoto, T., Tsutsui, T., Nishiguchi, A., Kobayashi, S., Tsukamoto, K., Saito, T., . . . Okamatsu, M. (2007). Preliminary evaluation of diagnostic tests for avian influenza using the Markov Chain Monte Carlo (MCMC) Method in an emergency surveillance. *Journal of Veterinary Medical Science, 69*(6), 673-675. https://doi.org/10.1292/jvms.69.673

Zhang, H., Peng, C., Duan, X., Shen, D., Lan, G., Xiao, W., . . . Chen, Y. (2014). A Cross-Sectional Study of Avian Influenza in One District of Guangzhou, 2013. *PLoS ONE, 9*(10), e111218. https://doi.org/10.1371/journal.pone.0111218

TABLE 1 True bird-level prevalence of H5 and H9 antibodies in birds sampled in backyard and commercial chicken production systems in Bangladesh (2016-2017).

Species	H5			H9		
	True Prevalence	95% CI		True Prevalence	95% CI	
		Lower	Upper		Lower	Upper
Backyard Chickens (N=576)	**3.7**	2.2	5.7	**15.7**	12.9	19.0
Backyard in-contact ducks (N=204)	**14.0**	9.5	19.6	**15.4**	10.8	21.1
Broiler chickens (N=954)	**1.0**	0.4	2.0	**1.0**	0.4	2.0
Unvaccinated layer chickens (N=800)	**7.4**	5.6	9.4	**5.3**	3.8	7.2
Vaccinated layer chickens (N=104)	**10.3**	5.2	17.8	**-**	-	-

FIGURES

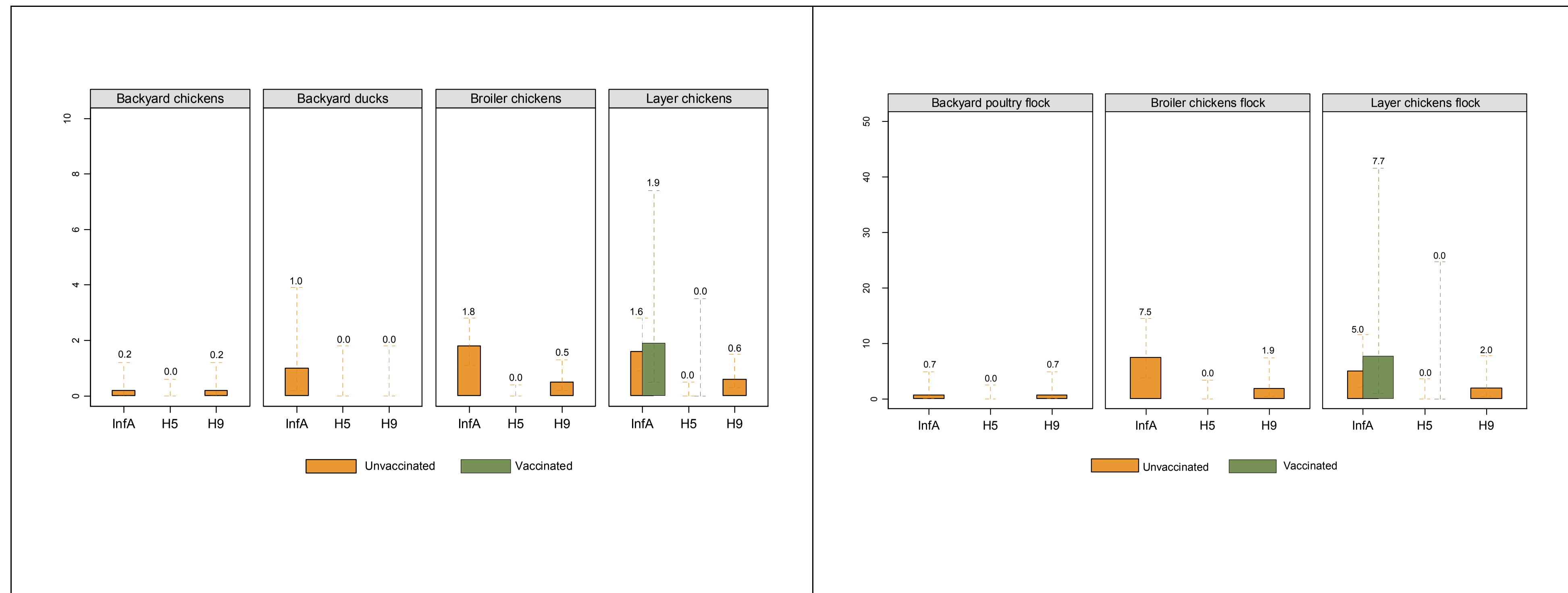


FIGURE 1 Bird-level (left panel) and flock-level (right panel) Influenza A (M-gene), H5 and H9 virus RNA prevalence detected by Reverse Transcription Polymerase Chain Reaction (RT-PCR) in backyard and commercial chicken production systems in Bangladesh (2016-2017). Data labels represent the apparent prevalence values. The confidence intervals are shown as dashed lines. Confidence intervals represent 95% limits if apparent prevalence was >0% and 97.5% limits if apparent prevalence was 0%.

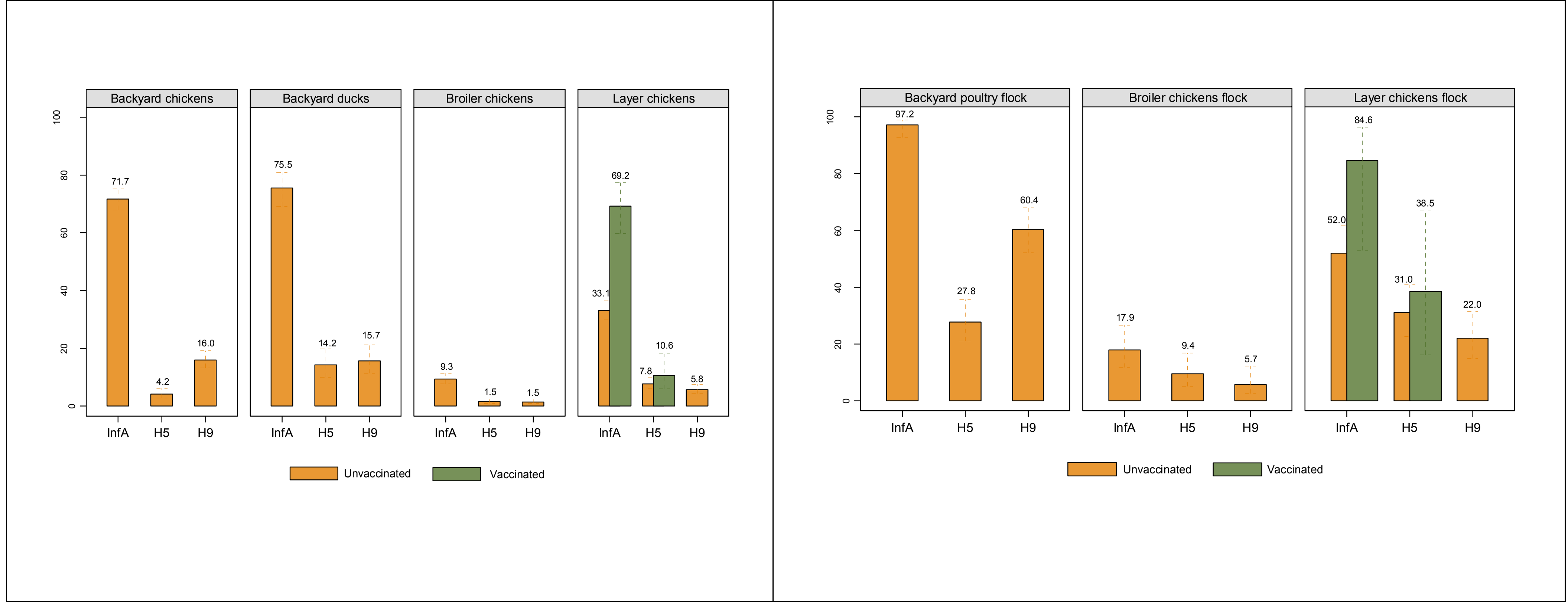


FIGURE 2 Bird-level (left panel) and flock-level (right panel) Influenza A, H5 and H9 antibody prevalence in backyard and commercial chicken production systems in Bangladesh (2016-2017). Influenza A antibodies were detected by Enzyme Linked Immunosorbent Assay (ELISA), and H5 and H9 antibodies were

detected by Haemagglutination Inhibition (HI) test (≥1/16 dilution). Data labels represent the apparent prevalence values. The 95% confidence intervals are shown as dashed lines.

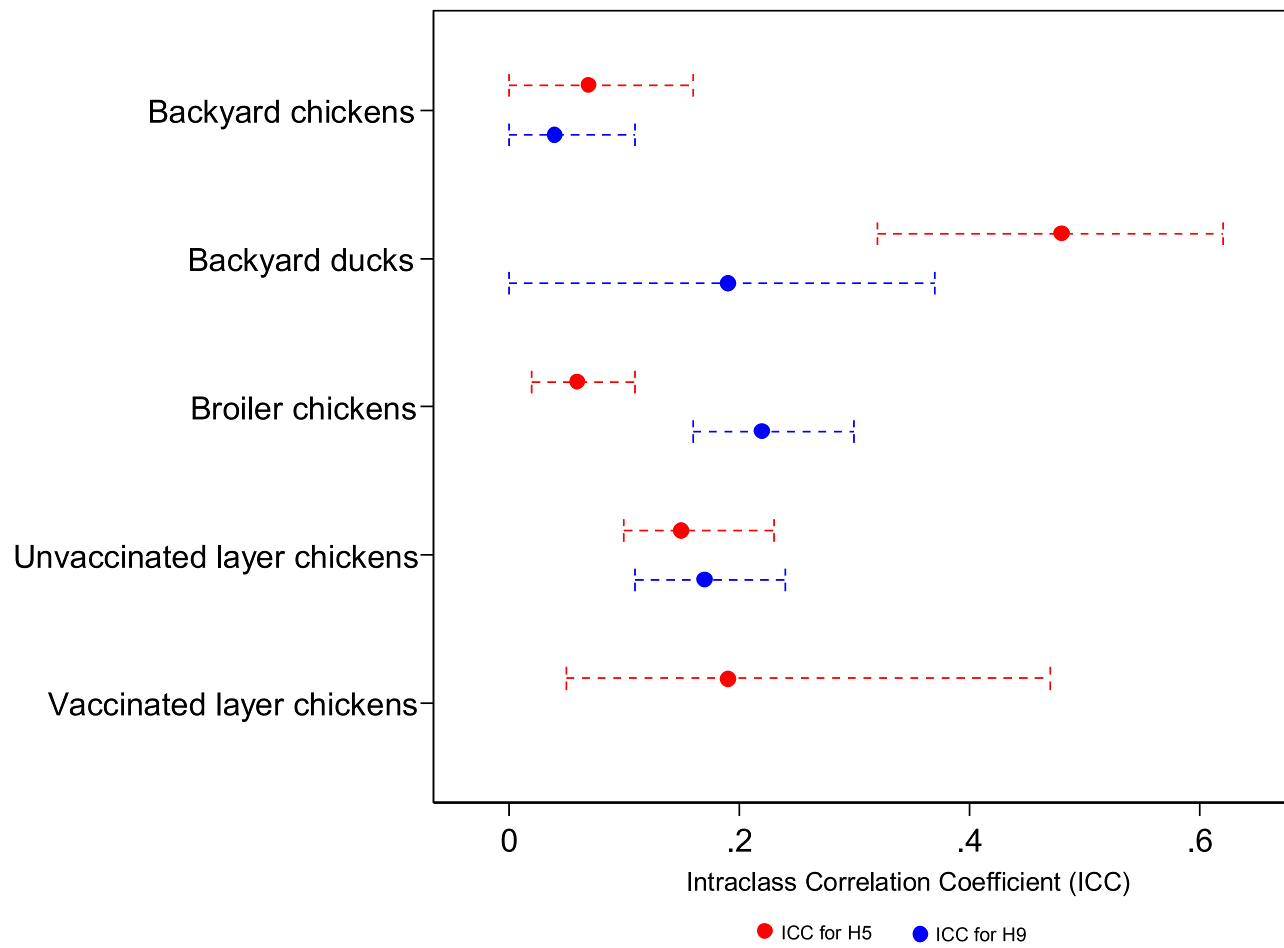
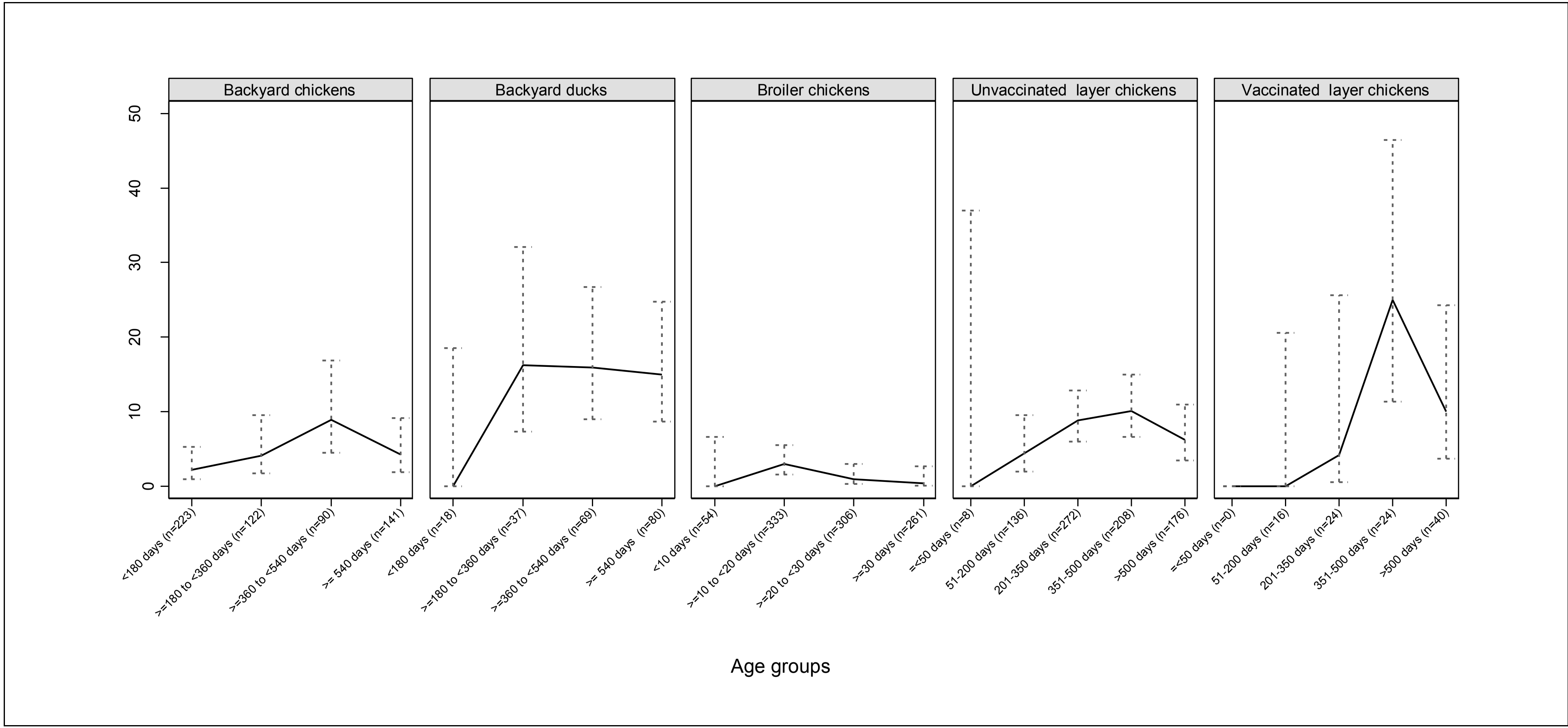


FIGURE 3 Intraclass Correlation Coefficients (ICCs) for H5 and H9 antibody positivity of birds within backyard and commercial chicken flocks in Bangladesh (2016-2017). The 95% confidence intervals are shown as dashed lines.



Backyard chickens
Backyard ducks
Broiler chickens
Unvaccinated layer chickens
Vaccinated layer chickens
0
10
20
30
40
50
<180 days (n=223)
>=180 to <360 days (n=122)
>=360 to <540 days (n=90)
>= 540 days (n=141)
<180 days (n=18)
>=180 to <360 days (n=37)
>=360 to <540 days (n=69)
>= 540 days (n=80)
<10 days (n=54)
>=10 to <20 days (n=333)
>=20 to <30 days (n=306)
>=30 days (n=261)
=<50 days (n=8)
51-200 days (n=136)
201-350 days (n=272)
351-500 days (n=208)
>500 days (n=176)
=<50 days (n=0)
51-200 days (n=16)
201-350 days (n=24)
351-500 days (n=24)
>500 days (n=40)
Age groups

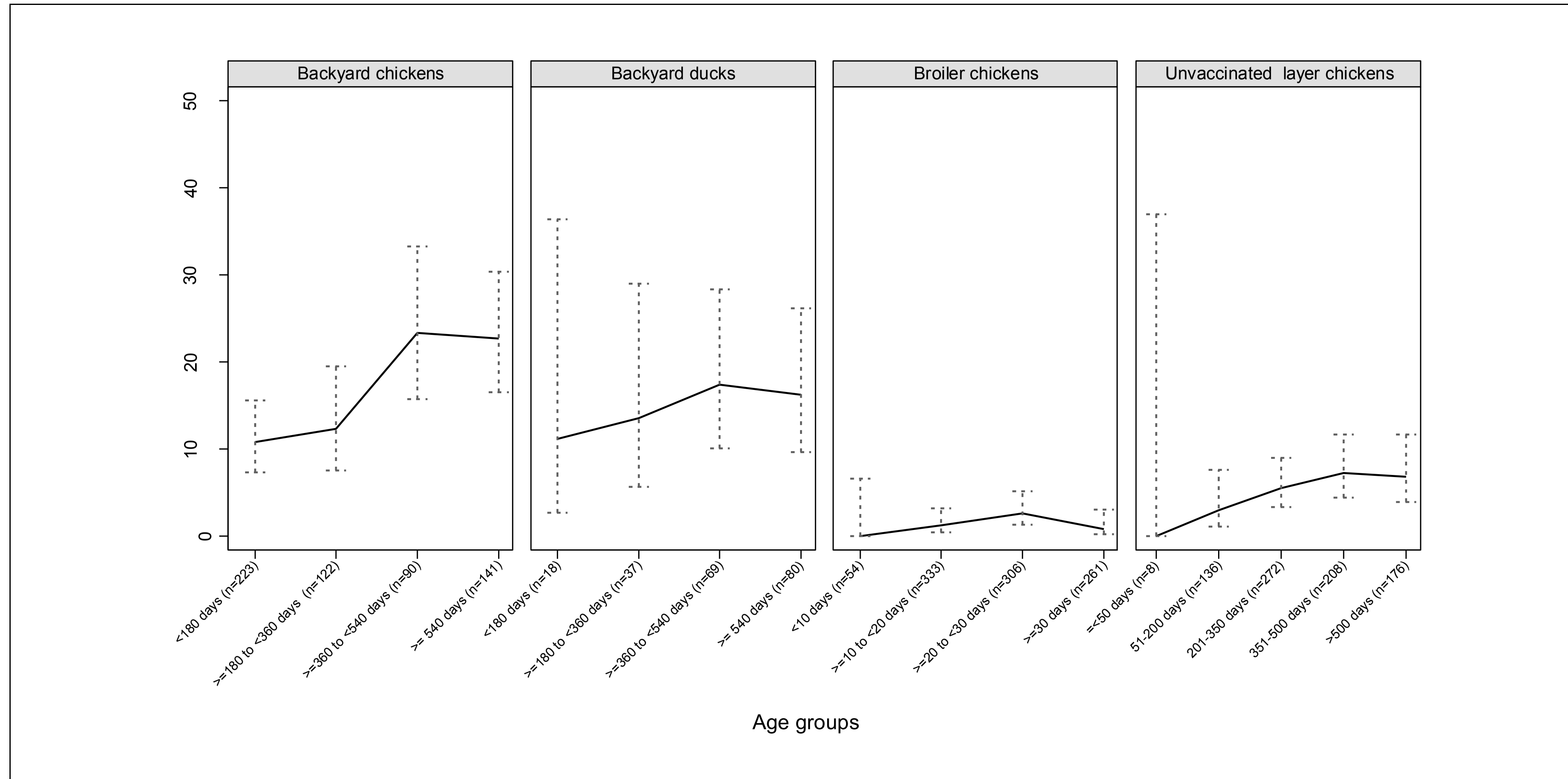


FIGURE 4 Bird-level H5 (upper panel) and H9 (lower panel) antibody prevalence by age group in backyard and commercial chicken production systems in Bangladesh (2016-2017). H5 and H9 antibodies were detected by Haemagglutination Inhibition (HI) test (≥1/16 dilution). Apparent prevalence is shown as a

solid line. Confidence intervals are shown as dashed lines. Confidence intervals represent 95% limits if prevalence was >0% and 97.5% limits if prevalence was 0%.

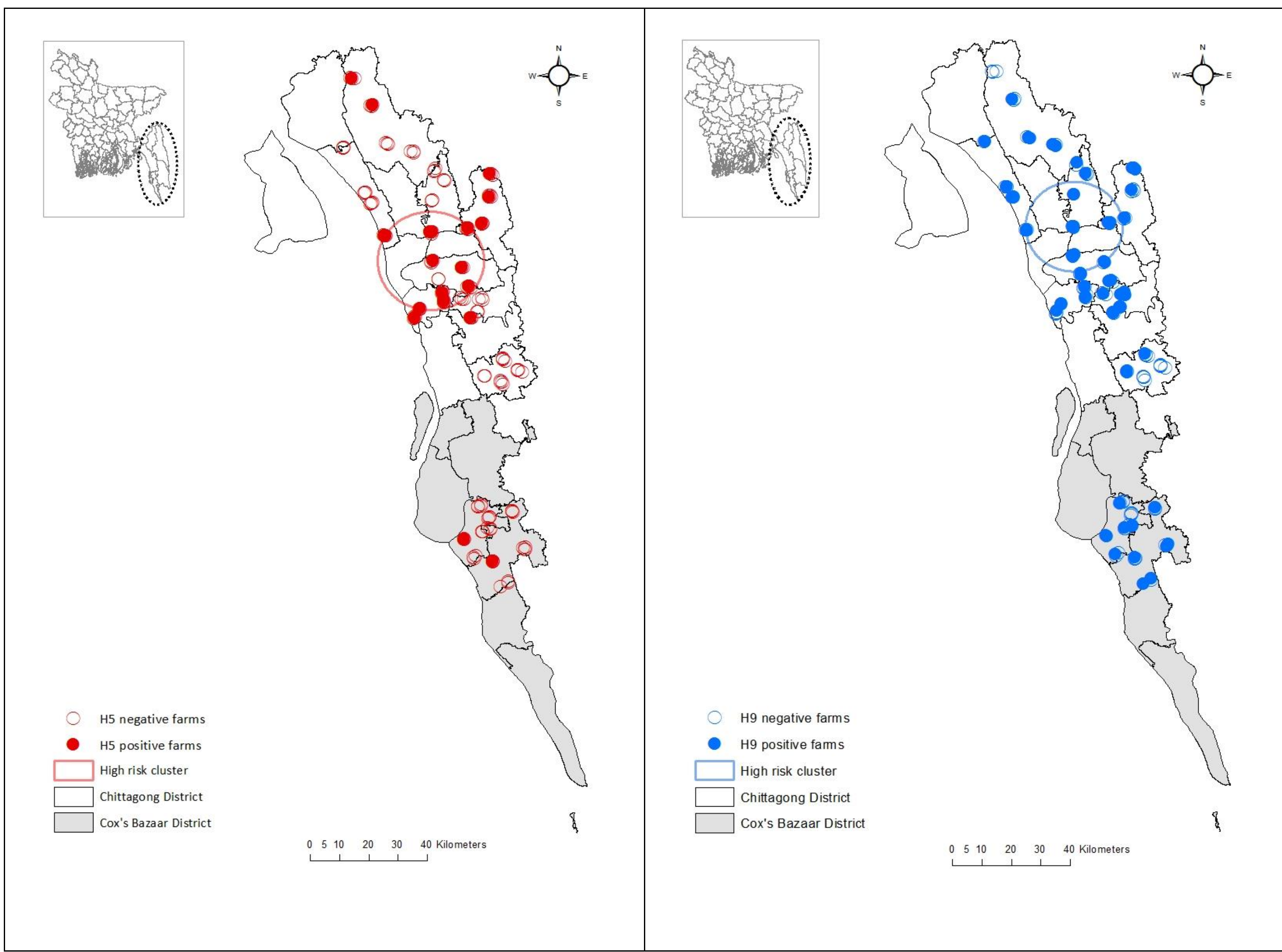

N
W
E
S
H5 negative farms
H5 positive farms
High risk cluster
Chittagong District
Cox's Bazaar District
0 5 10 20 30 40 Kilometers
N
W
E
S
H9 negative farms
H9 positive fams
High risk cluster
Chittagong District
Cox's Bazaar District
0 5 10 20 30 40 Kilometers

FIGURE 5 Spatial distribution of antibody positive and negative backyard farms in the Chittagong and Cox's Bazaar districts of Bangladesh (2016-2017), based on Haemagglutination Inhibition (HI) test results from backyard chickens. High-risk clusters for H5 (left panel) and H9 (right panel) antibody positivity in chickens are highlighted.

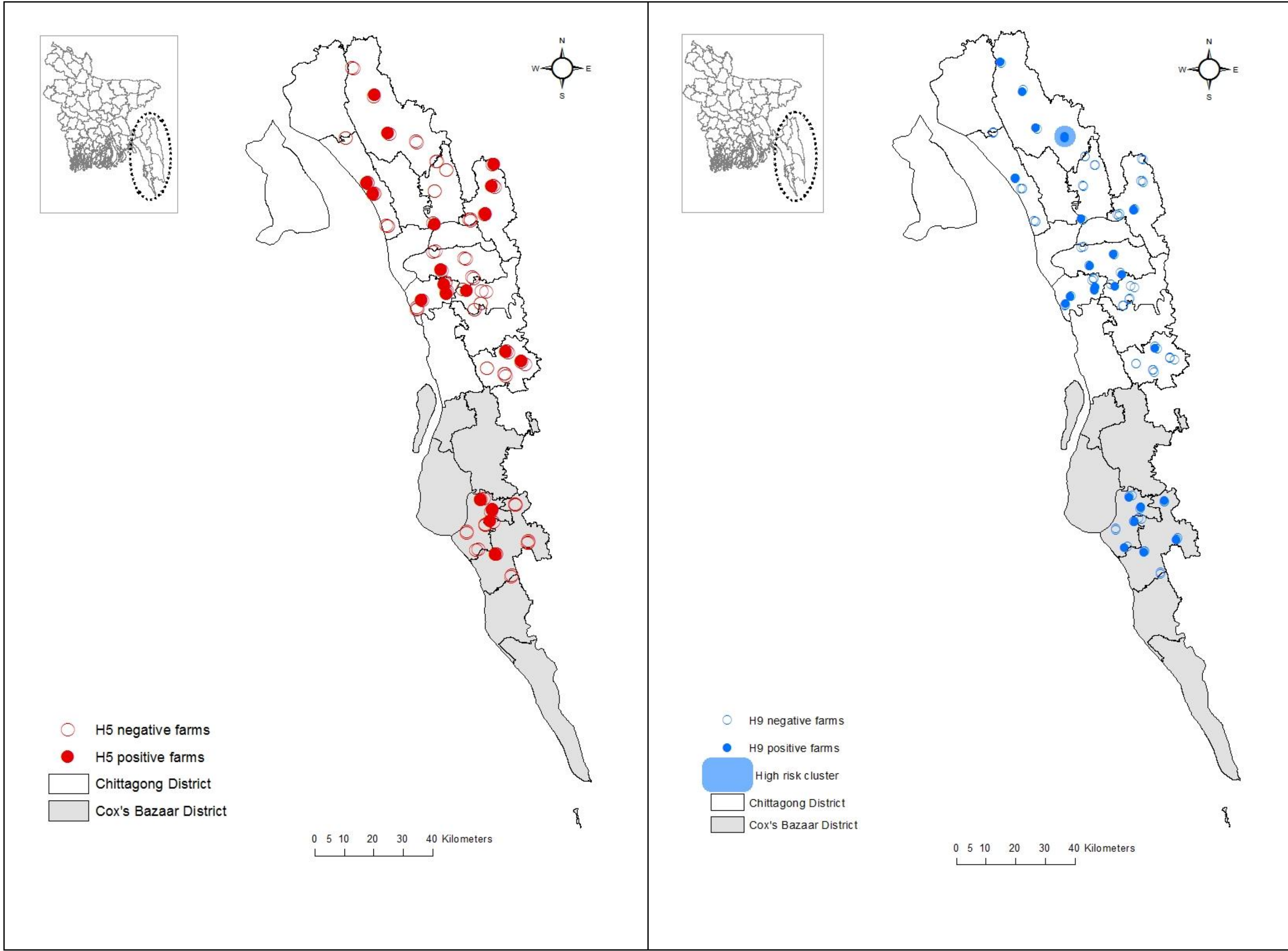

N
W
E
S
H5 negative farms
H5 positive farms
Chittagong District
Cox's Bazaar District
0 5 10 20 30 40 Kilometers
H9 negative farms
H9 positive farms
High risk cluster
Chittagong District
Cox's Bazaar District
0 5 10 20 30 40 Kilometers

FIGURE 6 Spatial distribution of antibody positive and negative backyard farms in the Chittagong and Cox's Bazaar districts of Bangladesh (2016-2017), based on Haemagglutination Inhibition (HI) test results from backyard ducks. A high-risk clusters for H9 (right panel) antibody positivity in ducks is highlighted.

SUPPORTING INFORMATION

SUPPLEMENTARY TABLE 1 Input parameters used for sample size calculations and estimated sample size for number of birds and farms to be sampled. Flock sensitivity was defined as the probability that at least one sampled bird in an infected flock is found to be positive, assuming that the flock is infected at a prevalence equal to or greater than the specified design prevalence (Sergeant & Perkins, 2015).

Parameters	Backyard chickens	Backyard in-contact ducks	Commercial broiler chickens	Commercial layer chickens
Input parameters for sample size calculations				
Test sensitivity (%)	98.0	98	98	98
Confidence level (%)	95.0	95	95	95
Design bird-level H5 antibody prevalence (%)	15.0	35.0	15.0	35.0
Design flock-level H5 antibody prevalence (%)	25.0	50.0	25.0	45.0
Flock size	10	3	1500	1500
Tolerance (%)	10.0	10	10	10
Minimum desired flock sensitivity (%)	65.0	95.0	75.0	95.0
Calculated flock sensitivity (%)	65.6	98.7	76.2	96.6
Estimated sample size				
Farms to be sampled	123	99	103	102
Birds to be sampled	4	2	9	8

SUPPLEMENTARY TABLE 2

Classification of the 14 sub-districts (upazillas) in the Chittagong district based on features identified to influence avian influenza viral transmission (Ahmed et al., 2012): 1) Density of backyard poultry farms, 2) Density of backyard chickens, 3) Location of the sub-districts within the district, 4) Environmental characteristics of the sub-district, and 5) Distance of the sub-district to Chittagong City, where most live bird markets are located.

Sub-district	Density of backyard poultry farms	Density of backyard chickens	Location of sub-district in the district	Environmental Characteristics					Distance (km) to Chittagong City	Selected for sampling
				Forests	Hills	Wide rivers	Small rivers/canals	Sea access		
Anowara	Quartiles 4	Quartiles 4	South West	Present	Present	Present	Present	Present	19.4	YES
Banshkhali	Quartiles 2	Quartiles 4	South	Present	Present	Present	Present	Present	48.0	NO
Boalkhali	Quartiles 4	Quartiles 1	Middle	Present	Present	Present	Present	Absent	17.3	NO
Chandanish	Quartiles 2	Quartiles 2	South East	Present	Present	Present	Present	Absent	33.8	YES
Fatikchari	Quartiles 1	Quartiles 2	North	Present	Present	Present	Absent	Absent	54.4	YES
Hathazari	Quartiles 4	Quartiles 1	North West	Present	Present	Present	Absent	Absent	21.7	NO
Lohagara	Quartiles 3	Quartiles 4	South	Present	Present	Absent	Present	Absent	59.4	YES
Mirsharai	Quartiles 1	Quartiles 3	North	Present	Present	Present	Absent	Present	58.0	NO
Patiya	Quartiles 4	Quartiles 1	Middle	Present	Present	Present	Present	Absent	24.8	YES
Rangunia	Quartiles 2	Quartiles 2	North East	Present	Present	Present	Absent	Absent	35.4	YES
Raozan	Quartiles 2	Quartiles 3	North East	Present	Present	Present	Absent	Absent	35.3	YES
Sandwip	Quartiles 1	Quartiles 2	Island	Present	Absent	Absent	Present	Present	67.5	NO
Satkania	Quartiles 3	Quartiles 4	South East	Absent	Absent	Present	Present	Absent	51.3	NO
Sitakunda	Quartiles 3	Quartiles 3	North West	Present	Present	Present	Present	Present	36.8	YES